Disease Markers

Epigenetic Biomarkers in Cancer

Lead Guest Editor: Yuen Y. Cheng
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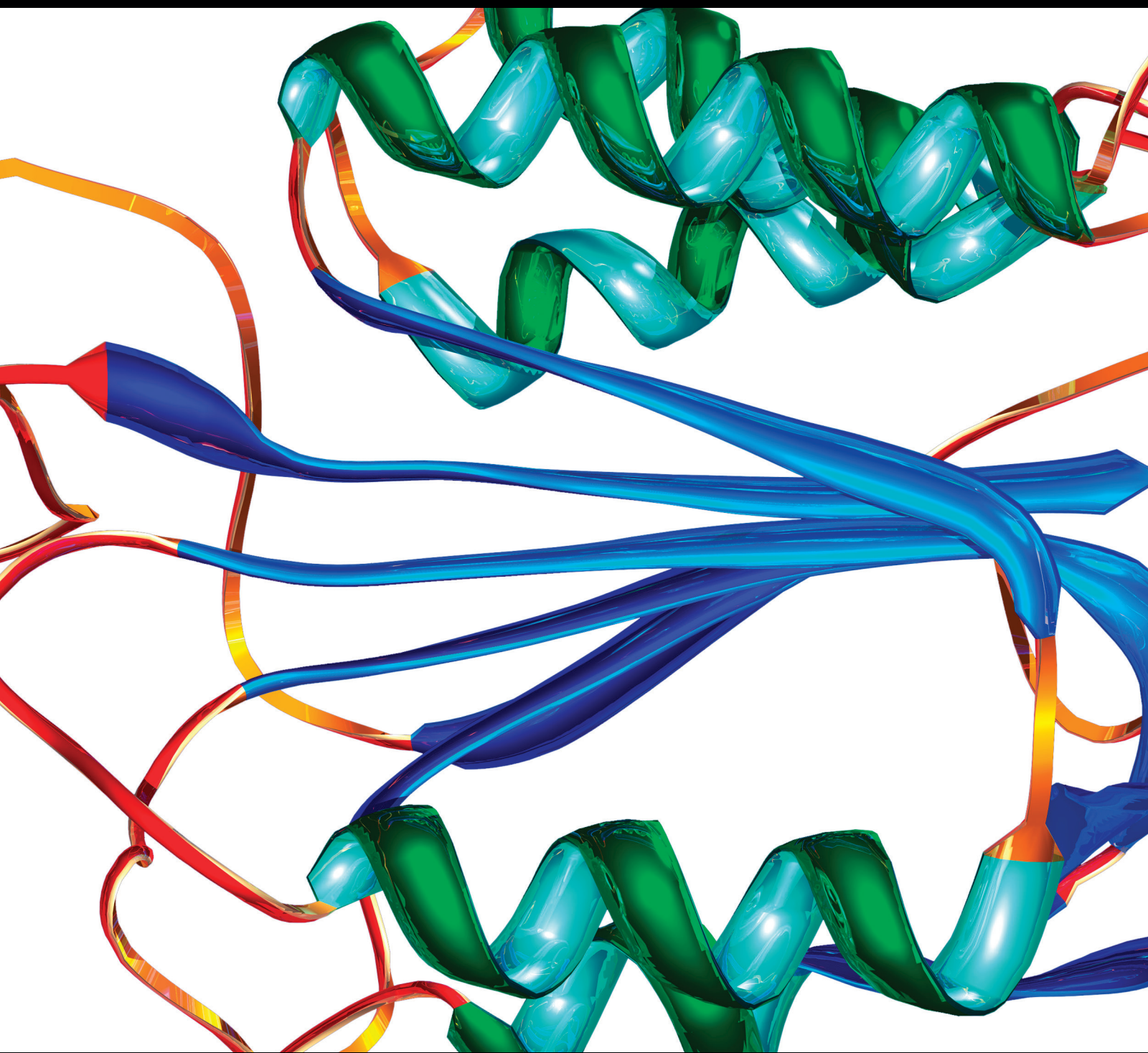

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Editorial Board

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Editorial

Epigenetic Biomarkers in Cancer

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We are pleased to announce the publication of this special issue focusing on epigenetic biomarkers in cancer. Epigenetics has gained the interest of researchers from all over the world; a total of 28 articles exist in this field that includes reviews and original research articles submitted for review. Among them, our editorial team, consisting of five prominent researchers in this field has selected four reviews and four original research articles for publication in this special issue. The reviews broadly cover the topics of DNA methylation, long noncoding RNAs, and single nucleotide polymorphisms, and the original research articles focus on DNA methylation, noncoding centromere RNAs, and mitochondria DNA. The selected articles highlight the progress in the field but suggest that there is also still a lack of translational linkage between epigenetic biomarker discoveries and their clinical application.

Exciting developments are occurring in the areas of early cancer detection, biomarker-based treatment selection, monitoring of disease response to treatment and early detection of recurrence. This is because research efforts are beginning to tap the wealth of significant and disease-driving information in DNA methylation and long noncoding RNA and in the complex and disease-influencing area of epigenetic modifications. In this special edition, we survey these exciting developments that are documented in review articles and novel experimental research results in specific, focused areas. Y. Y. Cheng et al. present novel findings suggesting DNA methylation as a novel noninvasive diagnostic marker for mesothelioma. Mesothelioma is a deadly cancer that requires ongoing efforts to understand its molecular drivers and find any effective molecular-based treatments. This novel research represents sorely needed progress in the molecular understanding of mesothelioma. C. Leygo et al. review the current state of research into the detection of epigenetic biomarkers of cancer using noninvasive samples from patients—an area that is hoped will soon revolutionise cancer detection and monitoring of response to therapy. D. Y. L. Chan et al. present cutting-edge results from live cell imaging showing the effects of transcribed noncoding RNA centromeric satellites in generating aberrant chromosome segregation during cell division—a valuable contribution in the significantly influential yet little-studied area of noncoding RNA. L. Bolha et al. review the research into the use of long noncoding RNAs (lncRNAs) from noninvasive body fluids as cancer biomarkers. This is an area of significant potential as we discover the richness of tissue and disease-specific information contained in lncRNAs and their ability for detection in sick patients by noninvasive means. In a focused, detailed study, S. Li et al. measure the prevalence of specific germline SNPs in a gene-influencing immune system interaction and their relationships to nasopharyngeal cancer susceptibility. G. M. Dexheimer et al. have carried out an extensive literature review of detection of epigenetic aberration in acute myeloid leukemia and myelodysplastic syndrome. This is a disease where aberrant DNA is highly accessible and thus ripe for exploitation in our efforts to

detect disease at earlier stages that are more successfully treated and to follow disease response and progression so as to more successfully modify treatments. J. Wu et al. use machine learning on TCGA methylation data to discover new biomarkers of lymph node metastasis in stomach cancer, having valuable predictive power for stomach cancer recurrence and treatment management for this fifth most common cancer in the world. H. Shuwen et al. carry out a review of current novel research that uses mitochondrial data—copy number variations, displacement loop mutations, variant burden, and microsatellite instability—to find more sensitive and accurate biomarkers in colorectal cancer. This is a timely review given controversies in this novel area of investigation.

The field of epigenetic regulation is an attractive area of research which was first studied in the 1970s and quickly gained global interest. Epigenetic regulation is involved in almost all developmental processes of mammalian cells from fertilisation, implantation, and differentiation during embryonic development to aging and carcinogenesis. Different stages of development are, thus, characterized by differences in epigenetic signatures, and variations in epigenetic patterns may also be associated with specific stages of disease. Carcinogenesis is a multistep process, and we believe that detection of changes in epigenetic profiles can be exploited to differentiate not only between different types of malignancies but also between different stages of cancer progression. Epigenetic biomarkers thus hold great promise to become more conclusive diagnostic and prognostic biomarkers for different cancers. DNA methylation is the most studied area of epigenetics, yet there are still only few methylation markers currently used in the clinical setting. We anticipate, however, that with the development of new technologies and due to their stability in cells and the circulation, DNA methylation biomarkers will play a prominent role in the near future. Further research is required in this field to ensure the widespread application of DNA methylation markers in clinical settings.

In conclusion, epigenetic regulation plays a major role in cancer formation, and analysis of epigenetic biomarkers has great potential to become clinically relevant. More studies are required to further develop and evaluate the clinical application of epigenetic biomarkers. This special issue brings together reviews and original articles focusing on both basic and translational research in the field of epigenetic biomarkers. We are confident that this special issue will advance the understanding and stimulate further research of epigenetic biomarkers in cancer.

Acknowledgments

We would also like to thank all the reviewers who have participated in the review process of the articles submitted to this special issue.

Yuen Yee Cheng
Hong Chuan Jin
Michael W. Y. Chan
Wai Kit Chu
Michael Grusch

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Research Article

SFRP Tumour Suppressor Genes Are Potential Plasma-Based Epigenetic Biomarkers for Malignant Pleural Mesothelioma

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Malignant pleural mesothelioma (MPM) is associated with asbestos exposure. Asbestos can induce chronic inflammation which in turn can lead to silencing of tumour suppressor genes. Wnt signaling pathway can be affected by chronic inflammation and is aberrantly activated in many cancers including colon and MPM. *SFRP* genes are antagonists of Wnt pathway, and *SFRP*s are potential tumour suppressors in colon, gastric, breast, ovarian, and lung cancers and mesothelioma. This study investigated the expression and DNA methylation of *SFRP* genes in MPM cells lines with and without demethylation treatment. Sixty-six patient FFPE samples were analysed and have showed methylation of *SFRP2* (56%) and *SFRP5* (70%) in MPM. *SFRP2* and *SFRP5* tumour-suppressive activity in eleven MPM lines was confirmed, and long-term asbestos exposure led to reduced expression of the *SFRP1* and *SFRP*2 genes in the mesothelium (MeT-5A) via epigenetic alterations. Finally, DNA methylation of *SFRP*s is detectable in MPM patient plasma samples, with methylated *SFRP2* and *SFRP5* showing a tendency towards greater abundance in patients. These data suggested that *SFRP* genes have tumour-suppresive activity in MPM and that methylated DNA from *SFRP* gene promoters has the potential to serve as a biomarker for MPM patient plasma.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and invariably fatal malignancy associated with asbestos exposure. The exact mechanism by which asbestos exposure leads to MPM carcinogenesis is not yet understood. It is known that asbestos is capable of inducing chronic inflammation and that chronic inflammation is capable of inducing tumour suppressor gene (TSG) silencing which is a driver of cancer. However, a much better understanding of the mechanism by which asbestos exposure leads to MPM is needed, so that the molecular players in the mechanism can be used as new molecular targets for diagnosis and treatment of MPM [1, 2].

It has been proposed that asbestos silicates attract and bind cations and that asbestos needles in the lungs will both retain the ions on the asbestos fibre surface and leach them into the cellular milieu [3], generating reactive oxygen species (ROS) and free radicals that begin the processes of cellular and DNA damage and genotoxicity, driving carcinogenesis [4, 5]. The high iron content of some asbestos fibres, as well as the propensity for asbestos to adsorb iron *in vivo*, has led some authors to suggest that iron-induced Fenton reactions contribute to increased ROS generation, inflammation, and carcinogenesis. It has been shown that asbestos exposure does not directly transform primary human mesothelial cells in tissue culture and instead induces inflammation [6–9]. Chronic inflammation caused by exposure of serosal surfaces to asbestos fibres is likely to represent a central factor in the carcinogenesis of MPM [10, 11].

The mechanism by which asbestos-induced chronic inflammation progresses to MPM carcinogenesis may be through epigenetic changes [10, 11]. A relationship between inflammation and promoter DNA hypermethylation has been documented in many forms of cancer, including

MPM [11]. Dysregulation of epigenetic transcriptional control, as well as aberrant promoter DNA methylation and histone modifications in particular, is a fundamental feature of human malignancies [12]. Asbestos exposure may trigger MPM formation via this epigenetic DNA methylation route [10, 13–15], and thus, DNA methylation in MPM is an area of interest for investigation.

Global DNA methylation has been investigated in MPM, and a number of genes were found to be methylated at varying frequencies [10, 16, 17], with the extent of methylation correlating with self-reported asbestos exposure [16] and burden of asbestos fibres in the lung [10]. The level of promoter methylation in MPM cell lines was found to be higher than that found in normal mesothelial cell cultures, and higher methylation status was found in tumours compared with normal mesothelium [10, 16]. The extent of methylation in sarcomatoid MPM was greater for the less differentiated tumours for which prognosis is poorer than that seen in epithelioid MPM tumours, suggesting a link between DNA methylation increase and severity of MPM disease [17]. Methylation-induced silencing of tumour suppressor-like miRs has been observed in MPM, suggesting that aberrant DNA methylation is involved in MPM carcinogenesis [18]. Cell-free methylated promoter DNA from pleural effusion fluid has been used in the diagnosis of malignant pleural effusion for lung cancer [19], and methylated DNA in serum was shown to have prognostic significance in MPM [20]. Therefore, TSG promoter methylation may represent a potential diagnostic and prognostic biomarker for MPM and also a potential therapeutic target. Thus, this study investigated the expression and epigenetic changes to the *SFRP* family of TSGs.

SFRPs belong to the family of Wnt pathway antagonists, and the *SFRPs* were found to be silenced in colon, gastric, breast, and lung cancers, with some members silenced in MPM [21–25]. The Wnt signaling pathway is of particular interest because comprehensive studies have shown that this pathway is involved in many cancers. The Wnt pathway is activated in MPM [26], and inhibition leads to apoptosis in gastric cancer cells [21]. Silencing of *SFRP4* and *SRFP5* has been linked to pemetrexed [26] and cisplatin [22] resistance, two drugs used in standard MPM treatment. Epigenetic silencing of the Wnt pathway is well characterized in colon cancer, a cancer known to be related to chronic inflammation. Recently, it was shown that the downregulation of *SFRP* gene family members in gastric and colorectal cancer is mediated by methylation silencing [21, 27]. Thus, downregulation of *SFRP* genes may represent a mechanism of aberrant Wnt signaling activation [21, 27].

In this study, we studied the mRNA expression and methylation of *SFRP*s in a panel of MPM cell lines. We also adopted an *in vitro* asbestos exposure model using the immortalised mesothelial cell line Met-5A and studied the methylation status and mRNA expression of members of the known tumour suppressor *SFRP* gene family. The *SFRP* methylation status in a cohort of 66 MPM patient DNA from FFPE samples was analysed using methylation-specific primers (MSP). Functional significance of *SFRP2* and *SFRP5* was studied in 11 MPM cell lines by cloning *SFRP2* and *SFRP5* into the cells to increase the expression of these genes and measure subsequent retardation in growth and colony formation of MPM cells. Finally, we established a detection method to study DNA methylation of *SFRP* genes in MPM and normal plasma samples with droplet digital PCR (ddPCR).

2. Materials and Methods

2.1. Cell Lines, Cell Culture, and Treatment. Five MPM cell lines (H2052, H2452, H28, H226, and MSTO) and the immortalised mesothelial cell line MeT-5A were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The primary mesothelioma cell line MM05 [28] was generated at the University of Queensland Thoracic Research Centre (The Prince Charles Hospital, Brisbane). Ren cells [29] were provided by the Laura Moro of the University of Piemonte Orientale A. Avogadro, Novara, Italy. VMC20, VMC23, and VMC40 were generated by The Medical University of Vienna. The primary MPM cell line 1137 was established at ADRI from an MPM patient biopsy. Cells were cultured at 5% CO_2, 37°C, and 95% humidity in RPMI 1640 (MPM cells) or DMEM (MeT-5A). All media and FBS were from Life Technologies (Carlsbad, CA, USA).

MeT-5A cells were cultured for 3 months in the presence or absence of chrysotile (1 $\mu g/cm^3$). *SFRP1* and *SFRP2* mRNA expression and promoter DNA methylation were analysed by quantitative reverse transcription PCR (RT-qPCR) and quantitative methylation-specific PCR (qMSP) as detailed below. MPM cells were seeded at 5×10^5 cells in a 10 cm^2 dish and treated 24 h later with 2 μM demethylating agent 5-aza-2′deoxycytidine (decitabine) every 24 h for 5 days. Cells were then harvested for DNA and RNA (detailed below).

2.2. Tumour Samples. The tumour samples used in this study are part of a previously reported MPM series to identify biomarkers [30]. All specimens were laser capture microdissected, guided by pathology marked tumour area. Ethics were obtained for this study through the Human Research Ethics Committee at Concord Repatriation General Hospital, Sydney. Written informed consent from all participants was obtained.

2.3. Analysis of DNA Methylation. Genomic DNA was extracted from MPM cell lines using the DNA Mini Kit (Qiagen, Valencia, CA, USA) and from MPM FFPE samples using the FFPE DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The methylation status of the *SFRP2* and *SFRP5* promoters in MPM cell lines was determined by MSP or qMSP as described previously [31]. Primers specific for methylated and unmethylated alleles in MSP were as specified previously [21]. CpGenome universal methylated DNA (Millipore, Billerica, MA, USA) was used as a positive control for methylation, and normal buffy coat (BC) from a healthy donor was used as a control for the unmethylated locus in each amplification.

2.4. Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR). Total RNA was extracted from cell lines using

Trizol reagent (Life Technologies). Reverse transcription (RT) reactions were performed using 200 ng of total RNA with MMLV first strand cDNA kit (Promega, Madison, WI, USA) following the manufacturer's protocol. The expression of *SFRPs* mRNA was determined by RT-PCR or quantitative real-time PCR using the KAPA SYBR Fast qPCR Master Mix (Sigma) and VII7 QPCR System (Life Technologies). 18S was used as the reference gene. mRNA expression levels of SFRP genes were determined using the $2^{-\Delta\Delta Cq}$ method [32] with normalisation to the 18S gene.

2.5. Expression Plasmids and Transfection. The pcDNA3.1(+)*SFRP2* or pcDNA3.1(+)*SFRP5* expression construct was generated by PCR cloning, and the sequence was verified and subcloned into pcDNA3.1-TOPO expression vector (Life Technologies) as previously described [20]. Plasmids were introduced into cells by transfection with X-tremeGENE 9 DNA transfection reagent (Sigma) as per the manufacturer's instructions.

2.6. Immunofluorescence of SFRP2 and SFRP5 Expression. Cells transfected with an *SFRP2* or *SFRP5* expression construct or empty vector-transfected cells were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in PBS for 15 min, washed three times with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Fixed cells were blocked with PBS containing 0.1% Triton and 10% fetal bovine serum for 1 h at room temperature. For immunostaining, cells were incubated for 2 h at room temperature with rabbit anti-*SFRP2* or anti-*SFRP5* and mouse anti-β-actin antibody at 2.5 μg/mL (Abcam) in blocking solution. After three washes with PBS, cells were incubated for 1 h at room temperature with Alexa Fluor 596-labeled goat anti-rabbit antibody (Life Technologies) and Alexa Fluor 488-labeled goat anti-mouse antibody (Life Technologies). Nuclear counterstaining was performed with 0.5 μg/mL DAPI. Immunostained cells were observed under a fluorescence microscope.

2.7. Proliferation Assay. The rate of *in vitro* cell proliferation was assessed by quantifying increases in DNA measured by the SYBR green-based assay. MPM cells were transfected with an *SFRP2* or *SFRP5* expression construct or vector control in 96-well plates, every 24 hrs. One set of plates with medium was removed and the plates were frozen. Relative cell numbers were determined by staining with 200 μL/well of SYBR green I (Life Technologies) as described previously [31]. Proliferation was calculated and presented as a percentage of the intensity of control cells at 120 hrs. Each experiment was performed in triplicate.

2.8. Droplet Digital PCR. Primers for the amplification of methylated *SFRP* DNA via MSP were optimized using ddPCR EvaGreen (Bio-Rad) according to the manufacturer's recommendations. DNA isolated from 200 μL plasma samples was bisulfite converted as previously described [21], and 4 μL from a total of 50 μL converted DNA was used as a template for ddPCR. For a 20 μL ddPCR reaction, 2× EvaGreen ddPCR Supermix (Bio-Rad) and primers at a final concentration of 0.2 μM were used. Reactions were dispensed into each well of the droplet generator DG8 cartridge (Bio-Rad). Each oil compartment of the cartridge was filled with 70 μL of droplet generation oil for EvaGreen (Bio-Rad), and approximately 15000 to 20000 droplets were generated in each well with the use of the droplet generator (Bio-Rad QX200). The entire droplet emulsion volume (40 μL) was transferred onto a 96-well PCR plate (Eppendorf). The plate was then heat sealed with a pierceable foil in the PX1 PCR Plate Sealer (Bio-Rad) and placed in the thermocycler (Bio-Rad T1000). The thermal cycling conditions used were 95°C for 5 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final step at 72°C for 1 min. The reaction mixtures were then held at 4°C until needed. The cycled droplets were read individually with the QX200 droplet-reader (Bio-Rad) and analysed with QuantaSoft droplet reader software, version 1.7 (Bio-Rad). The error reported for a single well was the Poisson 95% confidence interval. The method of no template controls (NTC) was used to monitor contaminations and primer-dimer formation and to determine the cut-off threshold. Normal and plasma sample-positive populations were then used to calculate the positive number detected in the sample, and results were plotted as total copy number detected per sample.

2.9. Statistical Analysis. Student's *t*-test was used to compare the difference in proliferation of *SFRP2* or *SFRP5* and control-transfected cells, and $p < 0.05$ was taken as statistically significant for differences between the two groups. Overall survival (OS) was calculated from the time of surgery to the time of death or last follow-up. Methylation status of *SFRP2* and *SFRP5* was used to assess the association of DNA methylation with OS using the Kaplan-Meier method and Cox regression. Statistical calculations were carried out using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. SFRP Genes Are Silenced by DNA Hypermethylation in MPM Cell Lines and Tumour Samples. *SFRP* genes are antagonists of the Wnt-signaling pathway and have been reported to be aberrantly activated in MPM [25, 26, 33]. Some members of the *SFRP* gene family are reported to be silenced via DNA methylation in MPM. Here, we confirm these studies by analyzing *SFRP* mRNA expression levels and DNA methylation status in seven cell lines. We first looked at mRNA expression of *SFRP1*, *SFRP2*, *SFRP4*, and *SFRP5* in MPM cell lines; *SFRP3* was not included as it does not have a distinctive CpG island. We compared the mRNA expression of *SFRP* genes in MPM cell lines to the noncancer MeT-5A and found that for most of our MPM cell lines *SFRP* genes are downregulated (Figure 1(a)). We treated these MPM cell lines with a demethylating agent (decitabine). Results indicated that expression of at least two *SFRP* genes was reactivated in every MPM cell line (Figure 1(a) right), which is a strong indication of DNA hypermethylation. We then analysed DNA methylation status in MPM cells. We narrowed down our focus to *SFRP2* and *SFRP5*, because for most of the MPM cell lines, there was a very low or no baseline detection of *SFRP*2 and *SFRP*5 (Figure 1(a)).

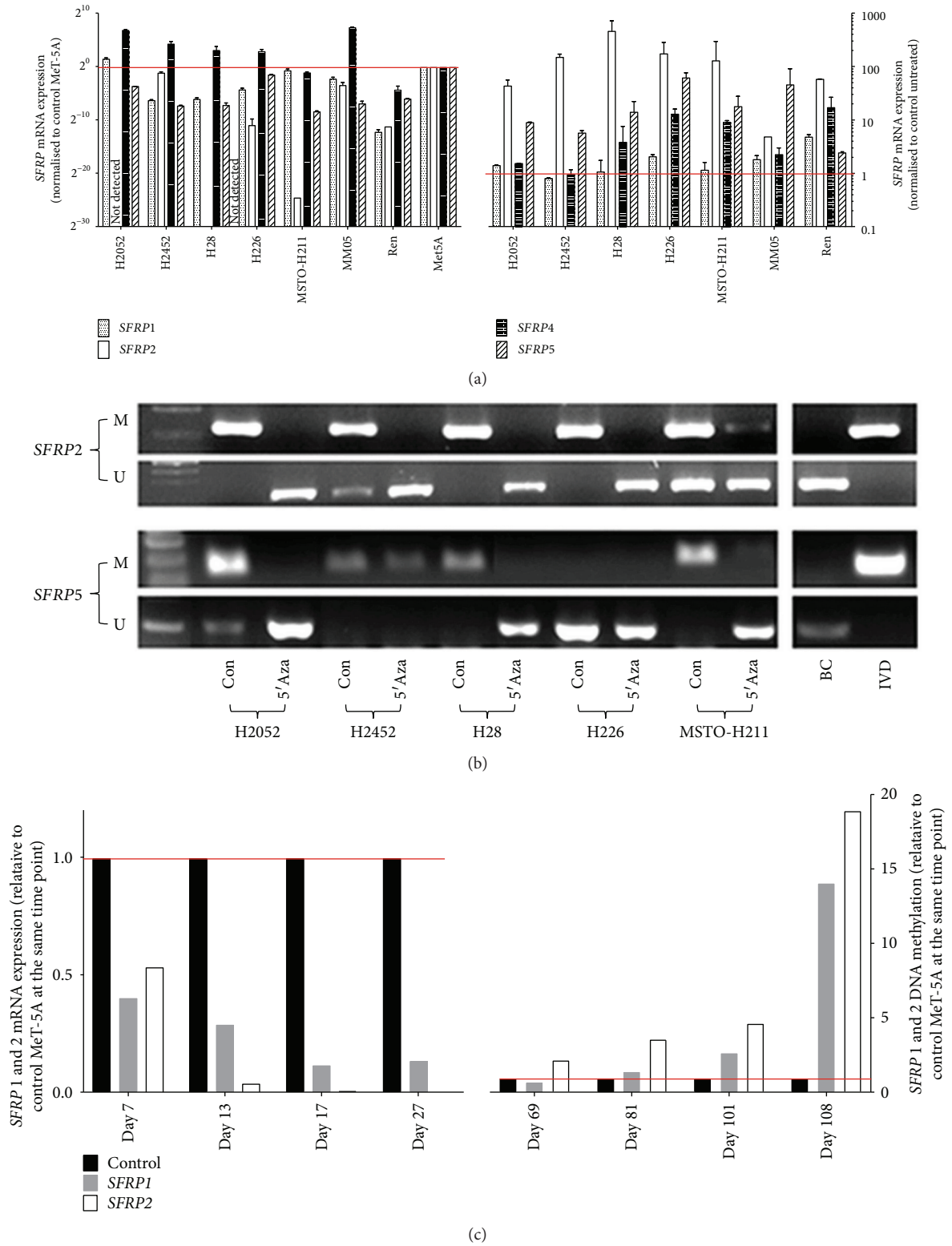


Figure 1: Asbestos-induced downregulation of tumour suppressor gene expression due to DNA methylation. (a) Basal expression and demethylated expression of mRNA of *SFRP* genes were determined by RT-qPCR in 7 MPM cell lines and in MeT-5A. Results were normalized to 18S and are expressed relative to the expression of MeT-5A or control untreated cells. (b) The methylation status of *SFRP2* and *SFRP5* were determined by MSP cell lines. (c) The expression of *SFRP1* was determined by RT-qPCR and DNA methylation by qMSP in MeT-5A cells with or without asbestos exposure. mRNA expression or methylation status was presented as fold change to parental untreated MeT-5A cells. (M = methylated; U = unmethylated, BC = buffy coat isolated from a healthy donor, IVD = universal methylated DNA control).

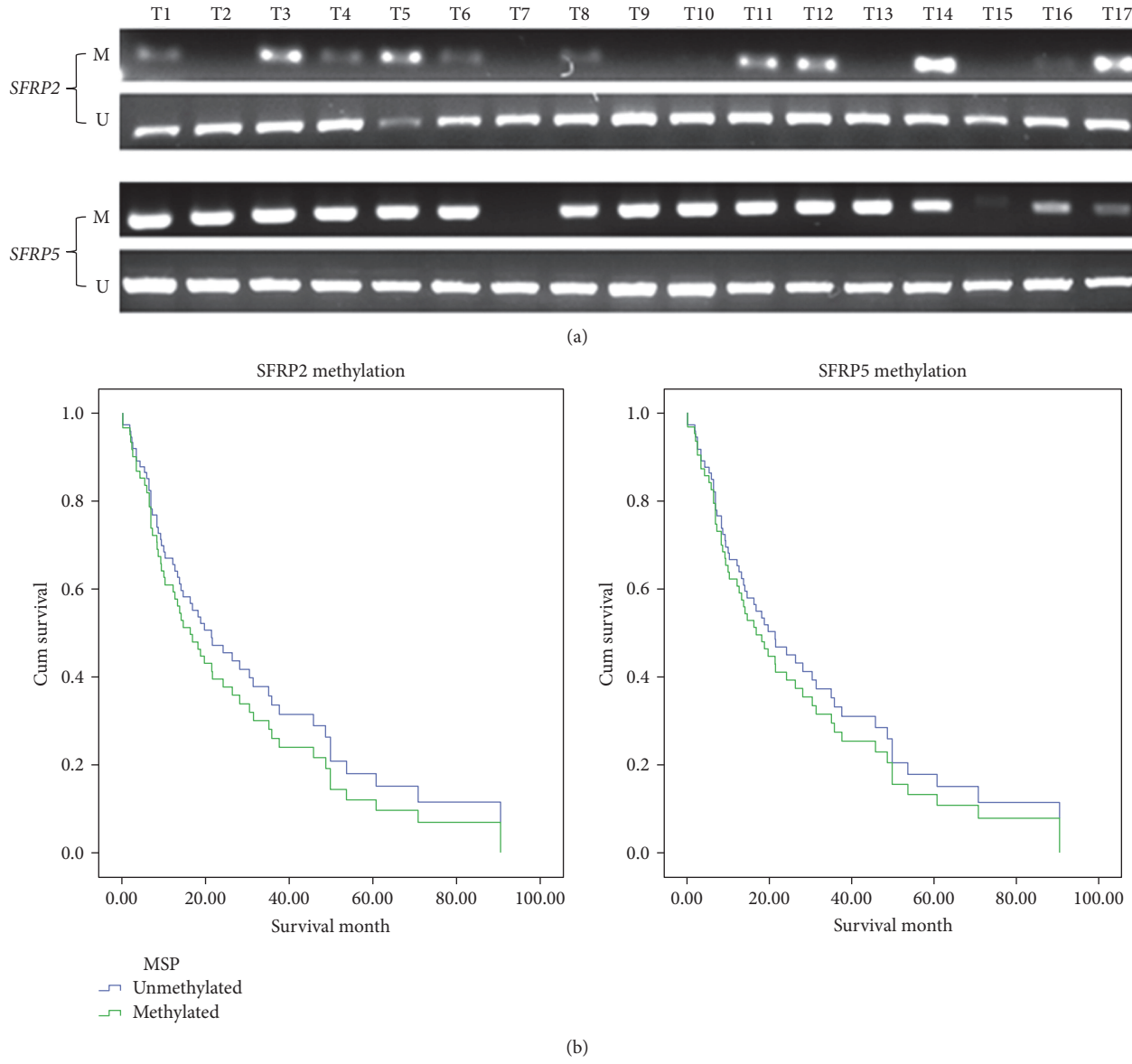


Figure 2: *SFRP2* or *SFRP5* methylation in MPM FFPE samples and overall survival. (a) MSP of *SFRP2* and *SFRP5* using FFPE samples, shown on the gel are representative results. (b) Kaplan-Meier analyses of *SFRP2* (left) and *SFRP5* (right) using methylation results from FFPE samples.

Investigating the DNA methylation status of *SFRP2* and *SFRP5* in MPM cells lines revealed that *SFRP*2 and *SFRP*5 were consistently methylated and became unmethylated when treated with decitabine (Figure 1(b)). Our results reconfirm previous publications that *SFRP*s are downregulated through hypermethylation in MPM cells [33, 34].

3.1.1. The Effect of Asbestos Exposure on Tumour Suppressor Genes SFRP1 and SFRP2. We next studied the relationship between chronic asbestos exposure and tumour suppressor gene regulation. We speculated that the mechanism of asbestos-induced carcinogenesis could involve the suppression of tumour suppressor genes by DNA hypermethylation, as it is known that DNA methylation plays a major role in carcinogenesis [21, 31, 35]. The mesothelial cell line MeT-5A was continuously exposed to low-level chrysotile asbestos (1 μg/cm^2) for 3 months. Low levels of chrysotile were in line with those not inducing apoptosis in previous studies but did induce ROS (data not shown). RNA and DNA were isolated from cells harvested at different time points. We selected *SFRP1* and *SFRP2* for mRNA expression and DNA methylation studies. In the above results, we observed high expression of *SFRP1* in the MeT-5A cell line. We also observed that *SFRP2* was highly upregulated after decitabine treatment. Therefore, we analysed mRNA expression and the DNA methylation status of the *SFRP1* and *SFRP2* genes following chronic asbestos exposure. Prior to asbestos exposure, *SFRP1* and *SFRP2* were highly expressed and unmethylated in MeT-5A. After asbestos exposure, *SFRP1* and *SFRP2* mRNA expression was progressively downregulated from day 7 to day 27 (Figure 1(c)). In these samples, we also observed low levels of DNA methylation (data not

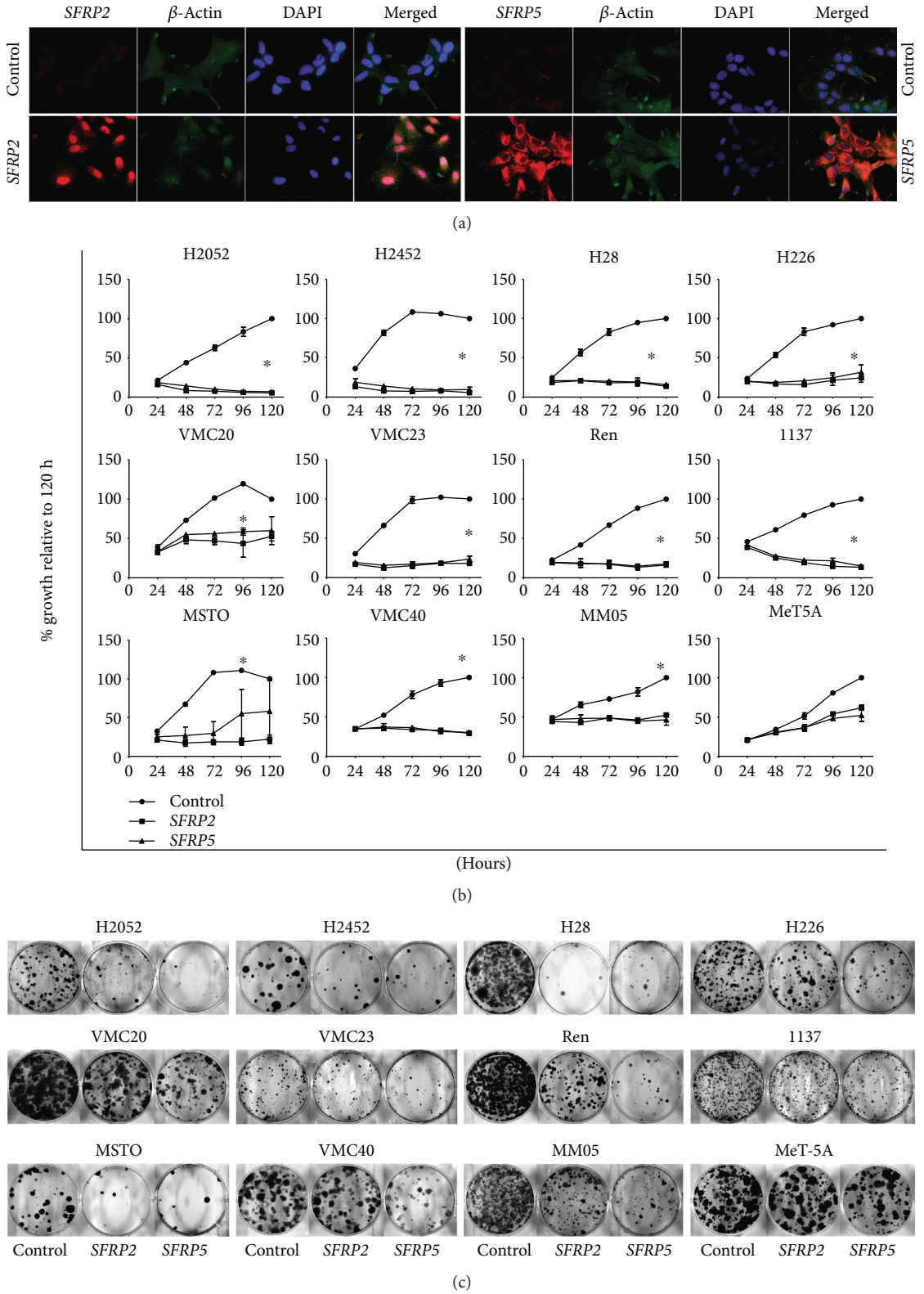
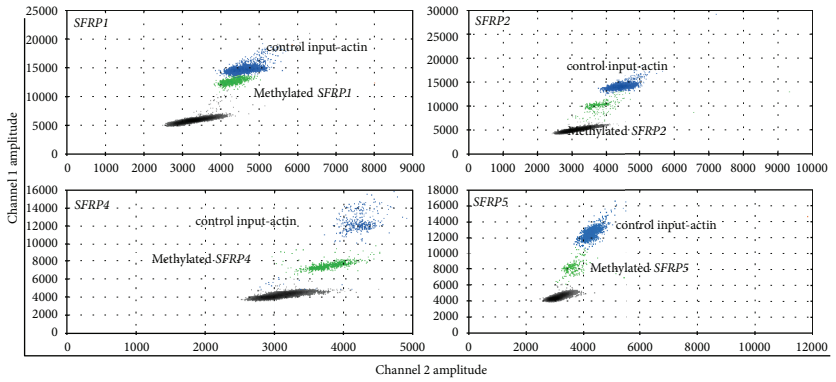
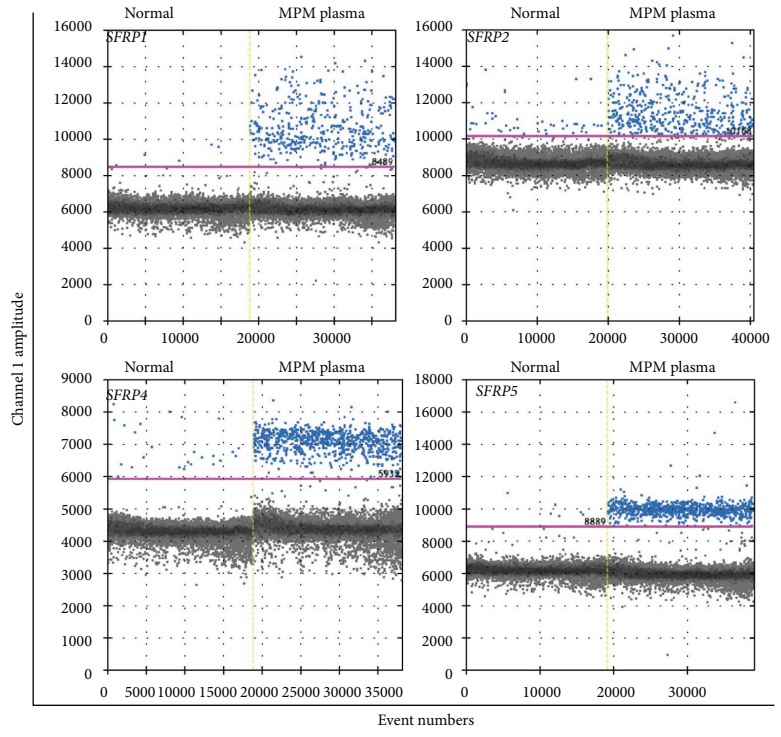


Figure 3: *SFRP2* or *SFRP5* re-expression in MPM cell lines inhibits cell growth and colony formation. (a) Protein re-expression of *SFRP2* and *SFRP5* was confirmed with immunofluorescence *SFRP2* or *SFRP5* red or β-actin in green and DAPI nuclear staining in blue. (b) 11 MPM cell lines and MeT-5A were transfected with pcDNA3.1 or pcDNA3.1-*SFRP2* or pcDNA3.1-*SFRP5*, and plates were harvested every 24 hrs for a total of 120 hrs. Cell proliferation was determined by SYBG assay, significant difference between *SFRP2* and *SFRP5* to control as $*$ with $p < 0.05$. (c) The clonogenic potential was assessed by plating 2500 transfected cells per 96 wells and then transferring to a 6-well plate at 24 hrs posttransfection, then incubated for a further 10–14 days. A representative picture from three independent experiments is shown.



(a)



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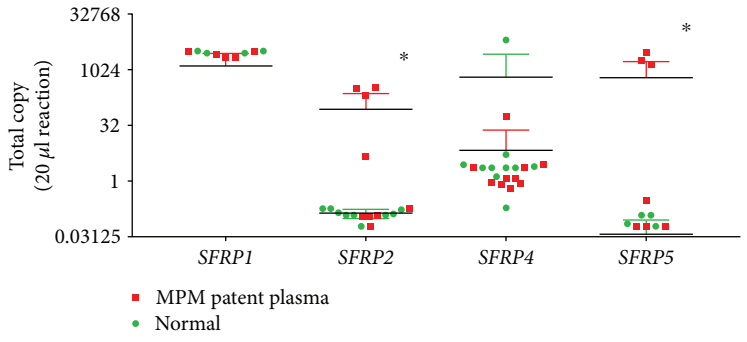

(c)

Figure 4: Detection of *SFRPs* in noninvasive plasma samples using droplet digital PCR. (a) Primers for confirmation of methylation of *SFRP* genes using cell lines as control with ddPCR EvaGreen assay. (b) Representative ddPCR results using noninvasive MPM plasma samples methylated or unmethylated fragments were detected using the same primer sets from Figure 4(a). (c) *SFRP* methylation in MPM and normal healthy control plasma was tested using the same ddPCR primer sets and conditions; result output was presented as total copy number detected per 20 μL ddPCR reaction. ddPCR results showed significant ($p < 0.05^*$) separation of normal (green) and MPM plasma samples (red) of *SFPR2* and *SFRP5*.

shown), and these methylation results were not significantly different between asbestos treatment and controls. We then measured the DNA methylation status of *SFRP1* and *SFRP2* in later samples by qMSP. Our qMSP results for *SFRP1* and *SFRP2* showed increased methylation from day 81 and becoming highly methylated by day 108 (Figure 1(c)).

This is the first time that exposure of MeT-5A nonmalignant cells to asbestos has been shown to cause downregulated mRNA expression and increased DNA methylation of *SFRP1* and *SFRP2*. Although asbestos is a known carcinogen and exposure is highly linked to development of mesothelioma, DNA methylation of *SFRP* genes has not previously been explored. Previous research hypothesised a link between chronic inflammation elicited by asbestos fibres in the mesothelial cavity, damaged scar tissue induced by the chronic inflammation, and inhibition by asbestos-induced ROS of repair of the scar tissue [36, 37]. Our results confirm our hypothesis that asbestos exposure induces an increase in DNA methylation of the promoter of the *SFRP1* and *SFRP2* tumour suppressor genes and downregulation of mRNA of those genes.

3.2. SFRP2 and SFRP5 Are Hypermethylated in MPM Tumour Samples. We next analysed the methylation status of *SFRP2* and *SFRP5* in 66 MPM patient samples because these two genes were the most frequently downregulated and methylated in our MPM cell lines. Representative methylation results are shown in (Figure 2(a)). Out of a total of 66 MPM samples, we found that 56% (40 out of 66) and 70% (46 out of 66) have methylation of *SFRP2* and *SFRP5*, respectively, with both *SFRP2* and *SFRP5* methylation detected in 44% (29 out of 66) samples. Methylation of *SFRP2* and *SFRP5* was not detected in samples from healthy normal donors. The effect of *SFRP2* or *SFRP5* DNA methylation on overall survival of MPM patients was assessed using the Kaplan-Meier method (Figure 2(b)). The Cox regression analysis indicated an association between overall survival and *SFRP2* ($p = 0.06$) and *SFRP5* ($p = 0.56$) DNA methylation status in our sample series. The lack of a significant correlation between overall survival of *SFRP2* and *SFRP5* DNA methylation in MPM contrasts with the situation in colorectal cancer, where *SFRP1* and *SFRP2* methylation has potential as an early diagnostic epigenetic biomarker [38].

3.3. The Functional Effects of SFRP2 and SFRP5 in MPM Cell Invasion and Colony Formation. Previous research has reported that the *SFRP* antagonists of the Wnt pathway are potential tumor suppressor genes and are frequently silenced and methylated in many cancers [21, 33, 34, 39]. In our study, we show that long-term exposure of asbestos in an *in vitro* model leads to downregulation of *SFRP* tumour suppressor gene expression and this downregulation is via DNA hypermethylation of their promoter regions. Our results for promoter methylation of *SFRP2* and *SFRP5* in MPM suggested that they are downregulated in MPM by hypermethylation and thus may have tumour suppressor potential in MPM. To investigate this, we cloned the ORF of both genes and transfected constructs containing these genes into MPM cells. Confirmation of overexpression of *SFRP2* and *SFRP5* is shown in Figure 3(a). In all 11 MPM cell lines tested, both *SFRP2* and *SFRP5* separately suppressed MPM cell growth compared to control transfections (Figure 3(b), $p < 0.05$). The immortalised MeT-5A cell line showed much less suppression when transfected and this was not significant (Figure 3(c), $p = 0.07$). Ectopic expression of *SFRP2* or *SFRP5* also inhibited the ability of MPM cells to form colonies (Figure 3(c)). Our results confirm that *SFRP2* and *SFRP5* are tumour suppressors of mesothelioma, similar to reports by others [33, 34].

3.4. Methylated SFRP2 and SFRP5 Promoter DNA Is Detectable in MPM Patient Plasma. As *SFRP2* and *SFRP5* are frequently methylated in MPM tumour samples, we speculated that the methylated DNA might be released by tumour cells and detectable in plasma. We employed recently available droplet digital PCR technology because it allows quantitative and sensitive detection of nucleic acids. We first optimized ddPCR condition with cell line samples (Figure 4(a)) and then applied this optimized method to the analysis of patient plasma samples (Figure 4(b)). Using the positive population shown in Figure 4(b), we then measured methylation status of *SFRP*s in samples from 10 MPM patients and 10 age-matched controls. We were able to detect methylated DNA from *SFRP2* and *SFRP5* with both showing a distinctive cut-off between patient and healthy normal plasma (Figure 4(c)). We were also able to detect *SFRP1 and SFRP4* methylation in plasma samples; however, there was no distinctive separation of patients and controls. There are many studies reporting detection of biomarkers in noninvasive samples from cancer patients, including sputum (lung cancer) [40], urine (bladder cancer) [41, 42], plasma (breast cancer) [43], and stool (colon cancer) [44, 45]. Although we have demonstrated that methylated *SFRPs* can be detected in plasma of MPM patients, the small sample size means that we are not able to make a definitive conclusion about whether we can use this finding as a diagnostic marker for MPM. These promising early data require validation in a larger series of samples from MPM patients and controls.

4. Conclusion

Our gene regulation, DNA methylation, cell growth, and colony formation results indicate that *SFRP2* and *SFRP5* both act as tumour suppressors of MPM and are silenced by DNA hypermethylation. *SFRP1* and *SFRP2* gene expression was downregulated by prolonged asbestos exposure in immortalised noncancer mesothelial cells. We also show that methylation of *SFRP2* (56%) and *SFRP5* (70%) is common in patient samples. The noninvasive detection of *SFRP2* and *SFRP5* in blood plasma samples demonstrates the potential of using DNA methylation status as a noninvasive epigenetic biomarker for MPM.

Conflicts of Interest

The authors declare no conflict of interest.

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References

[1] A. M. Marchevsky, N. LeStang, K. Hiroshima et al., "The differential diagnosis between pleural sarcomatoid mesothelioma and spindle cell/pleomorphic (sarcomatoid) carcinomas of the lung: evidence-based guidelines from the International Mesothelioma Panel and the MESOPATH National Reference Center," *Human Pathology*, vol. 67, pp. 160–168, 2017.

[2] H. H. Sun, A. Vaynblat, and H. I. Pass, "Diagnosis and prognosis-review of biomarkers for mesothelioma," *Annals of Translational Medicine*, vol. 5, no. 11, p. 244, 2017.

[3] A. Baldys and A. E. Aust, "Role of iron in inactivation of epidermal growth factor receptor after asbestos treatment of human lung and pleural target cells," *American Journal of Respiratory Cell and Molecular Biology*, vol. 32, no. 5, pp. 436–442, 2005.

[4] A. Aljandali, H. Pollack, A. Yeldandi, Y. LI, S. A. Weitzman, and D. W. Kamp, "Asbestos causes apoptosis in alveolar epithelial cells: role of iron-induced free radicals," *The Journal of Laboratory and Clinical Medicine*, vol. 137, no. 5, pp. 330–339, 2001.

[5] D. Upadhyay, V. Panduri, and D. W. Kamp, "Fibroblast growth factor-10 prevents asbestos-induced alveolar epithelial cell apoptosis by a mitogen-activated protein kinase-dependent mechanism," *American Journal of Respiratory Cell and Molecular Biology*, vol. 32, no. 3, pp. 232–238, 2005.

[6] L. Xu, B. J. Flynn, S. Ungar et al., "Asbestos induction of extended lifespan in normal human mesothelial cells: interindividual susceptibility and SV40 T antigen," *Carcinogenesis*, vol. 20, no. 5, pp. 773–783, 1999.

[7] M. Bocchetta, I. di Resta, A. Powers et al., "Human mesothelial cells are unusually susceptible to simian virus 40-mediated transformation and asbestos cocarcinogenicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 18, pp. 10214–10219, 2000.

[8] H. Yang, M. Bocchetta, B. Kroczynska et al., "TNF-α inhibits asbestos-induced cytotoxicity via a NF-κB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 27, pp. 10397–10402, 2006.

[9] A. L. Cleaver, K. Bhamidipaty, B. Wylie et al., "Long-term exposure of mesothelial cells to SV40 and asbestos leads to malignant transformation and chemotherapy resistance," *Carcinogenesis*, vol. 35, no. 2, pp. 407–414, 2014.

[10] B. C. Christensen, E. A. Houseman, J. J. Godleski et al., "Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome," *Cancer Research*, vol. 69, no. 1, pp. 227–234, 2009.

[11] S. P. Hussain and C. C. Harris, "Inflammation and cancer: an ancient link with novel potentials," *International Journal of Cancer*, vol. 121, no. 11, pp. 2373–2380, 2007.

[12] P. A. Jones and S. B. Baylin, "The fundamental role of epigenetic events in cancer," *Nature Reviews Genetics*, vol. 3, no. 6, pp. 415–428, 2002.

[13] S. Toyooka, H. I. Pass, N. Shivapurkar et al., "Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma," *Cancer Research*, vol. 61, no. 15, pp. 5727–5730, 2001.

[14] J. A. Tsou, L. Y. C. Shen, K. D. Siegmund et al., "Distinct DNA methylation profiles in malignant mesothelioma, lung adenocarcinoma, and non-tumor lung," *Lung Cancer*, vol. 47, no. 2, pp. 193–204, 2005.

[15] J. A. Tsou, J. S. Galler, A. Wali et al., "DNA methylation profile of 28 potential marker loci in malignant mesothelioma," *Lung Cancer*, vol. 58, no. 2, pp. 220–230, 2007.

[16] J. A. Tsou, J. A. Hagen, C. L. Carpenter, and I. A. Laird-Offringa, "DNA methylation analysis: a powerful new tool for lung cancer diagnosis," *Oncogene*, vol. 21, no. 35, pp. 5450–5461, 2002.

[17] Y. Goto, K. Shinjo, Y. Kondo et al., "Epigenetic profiles distinguish malignant pleural mesothelioma from lung adenocarcinoma," *Cancer Research*, vol. 69, no. 23, pp. 9073–9082, 2009.

[18] T. Kubo, S. Toyooka, K. Tsukuda et al., "Epigenetic silencing of microRNA-34b/c plays an important role in the pathogenesis of malignant pleural mesothelioma," *Clinical Cancer Research*, vol. 17, no. 15, pp. 4965–4974, 2011.

[19] H. Katayama, A. Hiraki, K. Aoe et al., "Aberrant promoter methylation in pleural fluid DNA for diagnosis of malignant pleural effusion," *International Journal of Cancer*, vol. 120, no. 10, pp. 2191–2195, 2007.

[20] J. R. Fischer, U. Ohnmacht, N. Rieger et al., "Promoter methylation of *RASSF1A*, *RARβ* and *DAPK* predict poor prognosis of patients with malignant mesothelioma," *Lung Cancer*, vol. 54, no. 1, pp. 109–116, 2006.

[21] Y. Y. Cheng, J. Yu, Y. P. Wong et al., "Frequent epigenetic inactivation of secreted frizzled-related protein 2 (SFRP2) by promoter methylation in human gastric cancer," *British Journal of Cancer*, vol. 97, no. 7, pp. 895–901, 2007.

[22] H. Y. Su, H. C. Lai, Y. W. Lin et al., "Epigenetic silencing of SFRP5 is related to malignant phenotype and chemoresistance of ovarian cancer through Wnt signaling pathway," *International Journal of Cancer*, vol. 127, no. 3, pp. 555–567, 2010.

[23] Y. Huang, T. M. Stewart, Y. Wu et al., "Novel oligoamine analogues inhibit lysine-specific demethylase 1 and induce reexpression of epigenetically silenced genes," *Clinical Cancer Research*, vol. 15, no. 23, pp. 7217–7228, 2009.

[24] T. Fukui, M. Kondo, G. Ito et al., "Transcriptional silencing of secreted frizzled related protein 1 (*SFRP 1*) by promoter hypermethylation in non-small-cell lung cancer," *Oncogene*, vol. 24, no. 41, pp. 6323–6327, 2005.

[25] H. Kohno, V. J. Amatya, Y. Takeshima et al., "Aberrant promoter methylation of WIF-1 and SFRP1, 2, 4 genes in mesothelioma," *Oncology Reports*, vol. 24, no. 2, pp. 423–431, 2010.

[26] B. He, A. Y. Lee, S. Dadfarmay et al., "Secreted frizzled-related protein 4 is silenced by hypermethylation and induces apoptosis in β-catenin-deficient human mesothelioma cells," *Cancer Research*, vol. 65, no. 3, pp. 743–748, 2005.

[27] M. Nojima, H. Suzuki, M. Toyota et al., "Frequent epigenetic inactivation of *SFRP* genes and constitutive activation of Wnt signaling in gastric cancer," *Oncogene*, vol. 26, no. 32, pp. 4699–4713, 2007.

[28] V. Relan, L. Morrison, K. Parsonson et al., "Phenotypes and karyotypes of human malignant mesothelioma cell lines," *PLoS One*, vol. 8, no. 3, article e58132, 2013.

[29] W. R. Smythe, L. R. Kaiser, H. C. Hwang et al., "Successful adenovirus-mediated gene transfer in an in vivo model of human malignant mesothelioma," *The Annals of Thoracic Surgery*, vol. 57, no. 6, pp. 1395–1401, 1994.

[30] M. B. Kirschner, Y. Y. Cheng, N. J. Armstrong et al., "MiR-score: a novel 6-microRNA signature that predicts survival outcomes in patients with malignant pleural mesothelioma," *Molecular Oncology*, vol. 9, no. 3, pp. 715–726, 2015.

[31] Y. Y. Cheng, M. B. Kirschner, N. C. Cheng et al., "*ZIC1* is silenced and has tumor suppressor function in malignant pleural mesothelioma," *Journal of Thoracic Oncology*, vol. 8, no. 10, pp. 1317–1328, 2013.

[32] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[33] A. Y. Lee, B. He, L. You et al., "Expression of the secreted frizzled-related protein gene family is downregulated in human mesothelioma," *Oncogene*, vol. 23, no. 39, pp. 6672–6676, 2004.

[34] V. Perumal, A. M. Dharmarajan, and S. A. Fox, "The Wnt regulator SFRP4 inhibits mesothelioma cell proliferation, migration, and antagonizes Wnt3a via its netrin-like domain," *International Journal of Oncology*, vol. 51, no. 1, pp. 362–368, 2017.

[35] Y. Y. Cheng, H. Jin, X. Liu et al., "Fibulin 1 is downregulated through promoter hypermethylation in gastric cancer," *British Journal of Cancer*, vol. 99, no. 12, pp. 2083–2087, 2008.

[36] G. Liu, P. Cheresh, and D. W. Kamp, "Molecular basis of asbestos-induced lung disease," *Annual Review of Pathology: Mechanisms of Disease*, vol. 8, no. 1, pp. 161–187, 2013.

[37] A. Kuraishy, M. Karin, and S. I. Grivennikov, "Tumor promotion via injury- and death-induced inflammation," *Immunity*, vol. 35, no. 4, pp. 467–477, 2011.

[38] J. J. Chen, A. Q. Wang, and Q. Q. Chen, "DNA methylation assay for colorectal carcinoma," *Cancer Biology & Medicine*, vol. 14, no. 1, pp. 42–49, 2017.

[39] M. S. Kim, J. Lee, and D. Sidransky, "DNA methylation markers in colorectal cancer," *Cancer Metastasis Reviews*, vol. 29, no. 1, pp. 181–206, 2010.

[40] A. J. Hubers, C. F. M. Prinsen, G. Sozzi, B. I. Witte, and E. Thunnissen, "Molecular sputum analysis for the diagnosis of lung cancer," *British Journal of Cancer*, vol. 109, no. 3, pp. 530–537, 2013.

[41] D. G. Ward, L. Baxter, N. S. Gordon et al., "Multiplex PCR and next generation sequencing for the non-invasive detection of bladder cancer," *PLoS One*, vol. 11, no. 2, article e0149756, 2016.

[42] P. F. Bassi, V. de Marco, A. de Lisa et al., "Non-invasive diagnostic tests for bladder cancer: a review of the literature," *Urologia Internationalis*, vol. 75, no. 3, pp. 193–200, 2005.

[43] A. P. Lourenco, K. L. Benson, M. C. Henderson et al., "A non-invasive blood-based combinatorial proteomic biomarker assay to detect breast cancer in women under the age of 50 years," *Clinical Breast Cancer*, vol. 17, no. 7, pp. 516–525.e6, 2017.

[44] T. Janz, K. Lu, M. R. Povlow, and B. Urso, "A review of colorectal cancer detection modalities, stool DNA, and fecal immunochemistry testing in adults over the age of 50," *Cureus*, vol. 8, no. 12, article e931, 2016.

[45] G. Russo, A. Patrignani, L. Poveda et al., "Highly sensitive, non-invasive detection of colorectal cancer mutations using single molecule, third generation sequencing," *Applied & Translational Genomics*, vol. 7, pp. 32–39, 2015.

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Review Article

DNA Methylation Events as Markers for Diagnosis and Management of Acute Myeloid Leukemia and Myelodysplastic Syndrome

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During the onset and progression of hematological malignancies, many changes occur in cellular epigenome, such as hypo- or hypermethylation of CpG islands in promoter regions. DNA methylation is an epigenetic modification that regulates gene expression and is a key event for tumorigenesis. The continuous search for biomarkers that signal early disease, indicate prognosis, and act as therapeutic targets has led to studies investigating the role of DNA in cancer onset and progression. This review focuses on DNA methylation changes as potential biomarkers for diagnosis, prognosis, response to treatment, and early toxicity in acute myeloid leukemia and myelodysplastic syndrome. Here, we report that distinct changes in DNA methylation may alter gene function and drive malignant cellular transformation during several stages of leukemogenesis. Most of these modifications occur at an early stage of disease and may predict myeloid/lymphoid transformation or response to therapy, which justifies its use as a biomarker for disease onset and progression. Methylation patterns, or its dynamic change during treatment, may also be used as markers for patient stratification, disease prognosis, and response to treatment. Further investigations of methylation modifications as therapeutic biomarkers, which may correlate with therapeutic response and/or predict treatment toxicity, are still warranted.

1. Introduction

Cancer is generally defined as a group of diseases governed by an accumulation of genetic mutations that are considered to be the major cause of uncontrolled cellular growth [1]. However, epigenetic mechanisms, which alter gene expression without affecting the genetic sequence itself, are also significantly involved in cancer development [2, 3]. Genetic modifications comprise mutations in tumor suppressor genes and oncogenes, both of which skew the balance towards dysregulated cellular proliferation. Epigenetic events are more complex, requiring modifications in chromatin structure or interference with RNA transcripts, and mostly include DNA methylation, histone modifications, nucleosome remodeling, and noncoding RNAs [4]. Thus, during the onset and progression of hematological malignancies, many changes can occur in the cellular epigenome, such as hypomethylation or increases in the methylation of CpG islands in promoter regions of key genes [5].

DNA methylation occurs by the addition of a methyl group (CH_3) to the 5′-carbon of cytosines that are followed by guanines (CpG sites), resulting in 5-methylcytosine (5-mC). This event is catalyzed by members of the DNMT (DNA methyltransferases) family, mainly DNMT1, DNMT3A, and DNMT3B. DNMT1 is localized in the replication fork during DNA replication, where the new DNA strand is

formed. Therefore, this enzyme binds to the daughter strand and methylates it to precisely mimic the original methylation pattern before replication [6, 7]. DNMT3A and DNMT3B present structural and functional similarities. These enzymes are able to introduce methylation into naked DNA, being associated with de novo DNA methylation and, thus, demonstrating an important role in normal development and disease [7, 8]. Methylation of promoter CpG islands usually occurs in or near promoter regions and may disturb the binding of transcription factors. This alone not only contributes to the regulation of gene expression but may also contribute to tumor suppressor gene silencing [9]. Not only that, loss of preserved epigenetic patterns can lead to activation or inhibition of different cellular signaling pathways, which can, invariably, lead to cancer, and it is known that genes that control cell cycle and DNA repair can be mutated or silenced by hypermethylation of their promoter sites [2, 10].

Several studies have already identified mutations in genes that encode crucial epigenetic regulators of gene transcription, such as *IDH1* (isocitrate dehydrogenase 1) and *IDH2* (isocitrate dehydrogenase 2), both of which catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate, *TET2* (ten eleven translocation 2) which is an α-ketoglutarate-dependent dioxygenase involved in the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and *DNMT3A*, all of which have been described in hematological malignancies [11–16]. Moreover, DNA methylation is maintained on subsequent cells by DNMT1, responsible for reproducing the parent strand's methylation pattern in the daughter strand [8], and mutations in the DNMT family are frequently described in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) [17–19], correlating with poor prognosis [20]. Recently, Spencer and collaborators described that hypomethylation is an initiating event in AML patients with the $DNMT3A^{R882H}$ mutation and DNMT3A-dependent CpG island hypermethylation occurs in consequence of disease progression [21].

The aim of this review is to demonstrate how DNA methylation acts as a potential biomarker for the diagnosis, management, and progression of hematological malignancies, focusing on myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).

1.1. Myelodysplastic Syndrome (MDS) and Acute Myeloid Leukemia (AML). MDS is a heterogeneous condition of clonal hematopoietic disorders characterized by ineffective erythropoiesis, dysplastic features, chromosomal abnormalities, and increased risk of AML progression. It presents a diverse phenotype, being stratified into low-risk or high-risk disease [22]. Hypomethylating agents (such as 5-azacitidine and 5-aza-2′-deoxycytidine, also known as decitabine), or thalidomide analogues (such as lenalidomide), are already employed for the treatment of MDS. Hematopoietic growth factors, immunosuppressive therapy, and hematopoietic stem cell transplantation are also employed, frequently as second-line therapy [23–25]. The choice of treatment is based on each patient's clinical parameters, such as karyotype, bone marrow blast percentage, and extent of cytopenia, among others [26]. Although bone marrow transplantation is the only choice offering a potentially curative treatment, few patients undergo this procedure because of their advanced age, medical comorbidities, and the limited availability of matching stem cell donors [27]. As supportive therapy, blood and platelet transfusions can be performed, as well as the use of iron chelators and antibiotics [23]. In spite of bone marrow transplantation being the only curative treatment described to date, a randomized phase III trial found that elderly patients with high-risk MDS, complex karyotype, and autosomal monocytes who were treated with decitabine showed higher progression-free survival when compared to patients receiving supportive therapy alone [28].

Decitabine inhibits DNA methylation and, at low doses, may reactivate silenced genes whereas, at high doses, may elicit cytotoxic effects. Studies are conflicting with regard to dose, treatment effectiveness, and patient eligibility, since hypomethylating agents, such as decitabine and 5-azacitidine, are widely used in the clinic even though they yield low complete remission rates, ranging from 15 to 20%. In this context, the treatment eligibility criteria are questioned, as the requirements for patient selection, dose strategy, and treatment duration are not clear, as well as latency and disease response or progression [29]. Dose comparison in decitabine treatments demonstrated more effectiveness at low doses [30]. However, low-dose treatments presented low efficacy and adverse toxic events when compared to treatment with cyclosporine in patients with low- or intermediate-risk MDS [31]. Therefore, a better understanding of response-to-treatment determinants is necessary to improve the therapeutic regimen with hypomethylating agents. In addition to the isolated pathology, other comorbidities should be evaluated in order to recognize which dose each patient should receive [32].

In general, MDS arises from abnormal gene expression, and this expression pattern will define the disease phenotype. Abnormal gene expression stems from different genetic mutations or epigenetic events, which can modify the expression levels of some genes. As an overall rule, these mutations induce cellular growth or inhibit apoptosis and may also block cellular differentiation, resulting in progression to acute leukemia [33]. In fact, the progression of MDS to AML is an example of the multistep theory of carcinogenesis. Kitamura and collaborators presented a new working hypothesis about the molecular bases of hematological malignancies employing the combination of mutations that could influence the phenotype and determine disease. Besides mutations that favor cellular proliferation and that block cellular differentiation, other phenomena in this multistep process were included, such as signal transduction events and epigenetic factors that are associated with dysregulated expression of genes, culminating in cellular immortalization, lack of differentiation, and increased cell survival and growth. Therefore, it is suggested that events that induce cellular immortalization and that favor a less differentiated phenotype are associated with the development of MDS; the addition of events that dysregulate cellular survival and

growth provides enough genetic advantages which allows the progression from MDS to AML [34].

AML is a heterogeneous disease, with different molecular signatures, therapeutic responses, and survival rates. It is a result of abnormal blast accumulation in the bone marrow, an event that, eventually, contributes to bone marrow failure. Blasts of the myeloid lineage are also found in peripheral blood at a concentration of approximately 20%. Different DNA methylation signatures have been described as markers for leukemogenesis and prognosis, and these also contribute to the understanding of disease development [35, 36].

Typically, AML treatment is divided into three phases: induction, consolidation, and maintenance. The rationale is to eliminate leukemic cells from the circulation with cytotoxic chemotherapy (induction) and then to eliminate residual leukemic cells from the circulation (consolidation and maintenance) [37]. Anthracyclines and cytarabine arabinoside (AraC) are the main drugs for most of the therapeutic regimens, aiming for complete remission and increased patient survival. Treatments utilizing a combination of these drugs show response rates with complete remission of 70 to 80% for patients under 60 years old [29, 30]. Refractory AML presents a therapeutic challenge, since standard treatment with AraC yields complete response rates of 17 to 20%. One clinical study for refractory AML aimed at achieving better treatment response by combining AraC with lenalidomide but did not present superior results when compared to AraC alone [29]. Moreover, complete remission in AML is generally not sufficient to increase overall survival [31, 32]. This, in part, can be explained by the fact that the presence of mutated genes in AML affects disease progression and prognosis stratifications, making it necessary to understand and validate its effects in order to assist in the clinical management of these patients [35].

Myelosuppression and febrile neutropenia are serious toxic events that arise during treatment and require great attention because of their effect on patient outcome [36, 37]. Although the use of small inhibitory molecules (such as imatinib and dasatinib) and monoclonal antibodies (such as rituximab) allow for longer treatments with lower toxicity rates, studies have already demonstrated that they may lead to serious grade 3 and 4 toxic events [38–40]. Therefore, it is important to establish optimal targets for each disease and to define when and how targeted therapies should be administered in order to establish a better and safer therapeutic regimen [41]. To this effect, determining the methylated genes that are associated with leukemogenesis and disease progression may also be important for selecting new therapeutic targets.

Comorbidities may also influence the therapeutic choices available, to the extent where some cases are considered ineligible for certain therapies because of previous or ongoing toxic events [42]. For patients older than 60 years of age, high-dose chemotherapy is poorly tolerated and treatment is rarely curative. Thus, treatment is directed towards increasing overall survival and quality of life [34]. This poses a challenge, and new approaches are needed in order to improve clinical outcome, contributing not only to better therapeutic responses, overall survival, and disease-free survival but also decreasing toxic events that may be fatal to the patient. Moreover, the development of new therapies demands time and incurs high costs [43]. Therefore, employing a molecular approach may optimize the existing therapeutic regimens, improving response rates, prognosis and, possibly, reducing toxic events.

2. Methods

The literature relating DNA methylation and staging/management of MDS and/or AML was reviewed and evaluated, with the goal of verifying which DNA methylation modifications, or changes in gene expression of epigenetic-modulating genes, were most present in disease onset, progression, staging, and toxic events.

The search terms were (biomarker or biomarkers) AND (DNA methylation) AND (acute myeloid leukemia) OR (myelodysplastic syndrome). Eligible literature was identified from PubMed, Science Direct, Web of Science, and Clinical Trial databases, and relevant data were extracted. Unpublished data, comments, letters, and conference proceedings were excluded from this search. A total of 65 articles and clinical trials with methylated genes (or mutations in epigenetic-modulating genes) suggested as marker for diagnosis, management, and prognosis of AML, and/or SMD patients were employed for this review.

3. DNA Methylation as an Epigenetic Biomarker

Cancer is characterized by its heterogeneity, given that each patient presents a variable molecular profile, which results in different molecular and physiological characteristics that contribute to development, prognosis, and response to treatment. In this context, the tumor microenvironment plays a fundamental role in which epigenetic components are associated with and contribute to tumorigenesis [44–47]. Epigenetic events, such as DNA methylation, are commonly identified in tumors, and these phenomena may aid in the understanding of the carcinogenic process since it is widely accepted that DNA methylation is related to cancer development and progression [48–51]. Moreover, these changes may be traced back and associated with disease staging and aggressiveness, allowing them to be employed as diagnostic and prognostic biomarkers. For this reason, studies seek to elucidate the interaction between these epigenetic modifications in chromatin remodeling, DNA replication and transcription, and the regulation of genes whose dysregulation is involved in carcinogenesis [52, 53].

Leukemias are a heterogeneous group of malignant neoplasms arising from the myeloid and/or lymphoid lineage, according to the dysplastic cell type, and which affects bone marrow, peripheral blood, and lymphoid tissues [54]. Aberrant epigenetic mutations have been demonstrated in different leukemia subtypes [48, 49, 55], and the number of identified changes is uprising, including genes involved in a plethora of signaling pathways and cellular processes [56, 57]. Association between epigenetic changes, such as DNA methylation, and clinical outcome among leukemia types suggests that these modifications should be explored

in order to develop a method that could improve patient stratification [55].

DNA methylation is an extensively studied epigenetic phenomenon, and different gene methylation patterns in tumor cells are used not only as markers for diagnosis but also as therapeutics targets. Different clinical trials have validated the ability of 5-azacytidine, a demethylating agent, in reducing global DNA methylation *in vivo* [58–60]. In this context, inhibitors of DNMT and histone deacetylases (HDAC) demonstrate clinical efficacy in treating hematological malignancies. Fandy and collaborators studied the methylation patterns of $p15^{INK4B}$ (cyclin-dependent kinase inhibitor 2B), a cell growth regulator; *CDH-1* (cadherin 1), a calcium-dependent cell-cell adhesion molecule; *DAPK-1* (death-associated protein kinase 1), a positive mediator of gamma interferon-induced programmed cell death; and *SOCS-1* (suppressor of cytokine signaling 1), which acts downstream of cytokine receptors participating in the negative feedback of cytokine signaling, in the bone marrow of 30 patients with MDS or AML. After treatment with 5-azacytidine and entinostat, an HDAC inhibitor, reversal of promoter methylation was observed but was not associated with clinical response [58]. In another study, administration of hypomethylating agents, such as decitabine, prior to allogeneic stem cell transplants improved patient outcome, all the while without increasing treatment toxicity in MDS patients [59]. The identification of factors that predict response to therapy could help increase treatment efficacy, while, at the same time, reducing its toxicity. For example, Achille and collaborators investigated global DNA methylation and gene expression of *CDKN2A* (cyclin-dependent kinase inhibitor 2A), *CDKN2B* (cyclin-dependent kinase inhibitor 2B), both regulators of the cell cycle at the G1 checkpoint; *HIC1* (transcriptional repressor 1), a growth regulatory molecule that acts as a tumor repressor; *RARB* (retinoic acid receptor beta), a retinoic acid nuclear receptor which also mediates cellular signalling, growth, and differentiation; *CDH1*; and *APAF1* (apoptotic peptidase activating factor 1), an apoptosis initiator by cleavage of caspase 9, before and during hypomethylating therapy, with the purpose of observing whether early changes could predict clinical response. Although global DNA methylation was not associated with clinical response, decreased *CDKN2A* promoter methylation was observed in patients achieving complete remission, and decreased *CDKN2B*, *RARB*, and *CDH1* promoter methylation was observed in responders [60].

In addition to these applications, DNA methylation can also be used as a biomarker for metastatic tumor screening [61, 62], cancer stage detection [63], malignant progression assessment [64], treatment response [65], and detection of minimal residual disease [66].

The importance of epigenetic modifications can be exemplified by the fact that patients who relapse after first-line therapy, or those stratified as high risk, may present lineage exchange, a phenomenon that occurs when an acute leukemia from the myeloid or lymphoid lineage at diagnosis presents a "switch" to the opposite lineage on relapse [67–70]. This process can be attributed to the original cellular clone, which may present morphological heterogeneity or high plasticity, or to a new leukemic clone. Hypotheses have already been raised in order to explain this event, but its mechanism has not yet been fully elucidated. However, since physiological plasticity is defined as the ability to modify a particular cellular target without altering its genotype, it may be inferred that epigenetic factors participate in mechanisms involved with phenotype regulation mechanisms and with responses to the cellular niche [67–70].

Since DNA methylation can alter gene function and drive malignant cell transformation, and because aberrant methylation modifications usually occur at an early stage of neoplastic development, different DNA methylation patterns may be investigated not only to identify markers for early tumor detection and risk stratification but also to predict treatment response and prognosis [71]. Several studies can be used to illustrate this application: Zhang and colleagues evaluated the clinical relevance of *DLX4* (distal-less homeobox 4) methylation, which plays a role in determining the synthesis of hemoglobin S, in patients diagnosed with MDS. It was found that this gene was significantly hypermethylated in MDS patients when compared to healthy controls. Moreover, patients with hypermethylated *DLX4* had a significantly shorter overall survival compared to patients with hypomethylated *DLX4* [72]. Similarly, *GPX3* (glutathione peroxidase 3) methylation, an enzyme that protects cells from oxidative damage, was identified in the bone marrow of patients diagnosed with MDS and AML, which associated with shorter overall survival compared to patients with unmethylated *GPX3* [73]. Wang and collaborators examined the methylation patterns of Wnt antagonist genes in 144 patients diagnosed with MDS. Survival analysis showed that methylated *sFRP1*, *sFRP4*, and *sFRP5* (secreted frizzled-related protein) were associated with a shorter overall survival. The frizzled-related family has a role in regulating cell growth and differentiation, besides modulating Wnt signaling through direct interaction [74]. In another study, Chaubey and colleagues investigated the effects of the methylation of the supressor of cytokine signalling gene (*SOCS-1*), a negative regulator of the cytokine pathway. A total of 100 patients diagnosed with MDS were evaluated, and methylation was observed in 53% of the cohort. Progression-free survival and median overall survival were shorter in patients in which *SOCS-1* was methylated, in comparison to those with unmethylated *SOCS-1* [75]. Overall, these studies present evidence that the methylation pattern of some genes may influence the course of disease, including with regard to prognosis and survival.

Generally, methylation patterns seem not to be directly related to general clinical data but have demonstrated a direct association to disease classification and stratification. For example, there are reports showing that methylation patterns were not different when compared to gender, age, tumor location, and other clinical parameters, such as white blood cell count [76–78]. Even so, it is important to investigate these methylation patterns across different clinical characteristics in order to observe if there are significant associations or correlations to clinical parameters. Therefore, there is still room to investigate methylation patterns as potential biomarkers for different lineages, as well as for predicting

prognosis, response to therapy, and/or toxicity to treatment. Many groups have investigated DNA methylation patterns in these contexts, and their findings are summarized in Table 1.

3.1. DNA Methylation as a Biomarker for Diagnosis and Prognosis. Epigenetic modifications, such as DNA methylation, may occur before histopathological changes and, for this reason, may be used as biomarkers for early diagnosis and risk assessment. It is important to note that many types of hematological malignancies are asymptomatic until they reach advanced stages, and, therefore, a thorough characterization of the biomarker is crucial in order for it to be employed for early detection and prediction of tumor progression [114].

Estrogen receptors (ER) regulated by DNA methylation have been reported to play a key role in leukemogenesis. In 40 patients diagnosed with leukemia and evaluated after one year of chemotherapy, it was observed that patients with *ER-α* methylation perceived no symptomatic relief, whereas patients without *ER-α* methylation obtained effective relief with treatment. This data suggest that methylation of *ER-α* could be further investigated as a biomarker for diagnosis and prognosis, since this gene is present in 95% of all evaluated leukemia cases and is related to a lower response to treatments directed towards symptom relief [90].

Methylation of *ID4* (inhibitor of DNA binding 4), a regulator of cell growth, senescence, differentiation, apoptosis, angiogenesis, and neoplastic transformation, was analyzed and suggested as a biomarker for the diagnosis of MDS. Li and collaborators analyzed the methylation status of 100 patients diagnosed with MDS, compared to 31 patients diagnosed with aplastic anemia (AA). *ID4* gene promoter methylation status correlated with clinical parameters in MDS and AA, and bisulfite analysis revealed that gene methylation was higher in patients diagnosed with MDS. Finally, the authors suggest that *ID4* gene promoter methylation could be a causative agent in hematopoietic disorders and, therefore, could be used to distinguish MDS from AA [96]. Similarly, Kang and colleagues investigated *ID4* gene methylation in two patients and in the demethylation-treated MDS cell line (MUTZ1) with bisulfite sequencing PCR. The two MDS patients were treated with decitabine and demonstrated, after treatment, a decrease in methylation. This indicates that this gene may be a biomarker for selection and assessment of effective therapeutic schemes [95].

DNA methylation has also been described as a biomarker for prognosis in hematological malignancies, allowing for a simpler and lower cost analysis than other genetic tests, and also aiding in therapeutic decisions [2, 115–118]. High levels of global DNA methylation are an independent adverse prognostic factor for MDS. Calvo and collaborators, for example, isolated DNA from bone marrow of patients at diagnosis and determined the methylation rate via ELISA. Patients with methylated DNA above 2.73% had a lower overall survival than those with levels below 2.73% and presented a negative trend in terms of leukemia-free survival [119].

Complement C1r (*C1R*) gene methylation, which encodes a protein that is involved in the complement system, has been shown to be a robust, simple, and cost-effective biomarker for prognosis investigation in 194 AML patients. A comparison of *C1R* DNA methylation with healthy donor samples and samples from patients diagnosed with AML showed that patients diagnosed with AML with favorable cytogenetic risk scores had higher methylation in *C1R* and longer overall survival. It was also suggested that DNA methylation of *C1R* might be of independent prognostic relevance; however, further studies must be carried out in order for this to be validated [76].

In another report, Kurtović and collaborators studied samples of newly diagnosed adults with AML, including de novo AML, secondary AML, AML occurring after MDS, and aplastic anemia presenting different cytogenetic patterns. The DNA methylation status of target promoter sequences of *p15* and O-6-methylguanine-DNA methyltransferase (*MGMT*), an enzyme involved in cellular defense against mutagenesis and toxicity from alkylating agents, was analyzed, and 81% of patients presented methylation in at least one of these two genes. It was not possible to prove that *p15* and/or *MGMT* could predict response to therapy and overall survival; however, it was found that AML patients with methylation in both genes or in *p15* alone had a higher frequency of early death and lower frequency of complete remission and presented a trend for shorter overall survival. Moreover, a cluster of abnormalities with adverse prognosis was observed in the group with aberrant methylation of both genes or of *p15* alone [77]. Thus, the methylation pattern of these genes may be used for AML patient stratification. In fact, the *p15* gene was associated with a tumor suppressor role based on its inactivation through hypermethylation of its promoter region in gliomas and leukemias [120]. In addition, this gene often exhibits hypermethylation in its promoter region in adults and children with both myeloid and lymphoid acute leukemia [121, 122].

Also with regard to prognosis, inhibition by methylation of the secreted frizzled-related protein genes *sFRP2* and *sFRP5*, both members of the Wnt pathway, was associated with poor prognosis in normal karyotype AML patients. The Wnt pathway is of great importance, since it plays an important role in the self-renewal of hematopoietic stem cells and in the development of progenitor cells [123]. In another study, Zhou and collaborators investigated the methylation status of the *GPX3* (glutathione peroxidase 3) gene promoter in the bone marrow of 110 MDS patients. Methylation was analyzed by methylation-specific PCR and bisulfite sequencing PCR and was observed in 15% of MDS patients. The methylation rate was higher than those of controls and lower than the methylation rate of AML patients. It was also observed that *GPX3* methylation was associated with older age, higher frequency of *DNMT3A* mutations, and shorter overall survival. The authors conclude that, therefore, *GPX3* methylation in bone marrow could be a marker for adverse prognosis and progression to leukemia in MDS patients [124].

3.2. DNA Methylation as a Biomarker for Treatment Response and Toxicity. Both AML and MDS are characterized by an exacerbated proliferation of undifferentiated myeloid cells [29]. Decitabine (5-aza-2′-deoxycytidine) or 5-azacytidine

Table 1: Methylated genes as markers for AML or MDS.

Gene	Disease	Patients (n)	Sample type	Associated factors	Ref.
AWT1	AML	356	BM/B	Classification of myeloid-derived leukemias. Hypermethylation could monitor the recurrence of disease during remission in patients undergoing allogeneic stem cell transfer.	[79]
BMI1	AML/MDS	54	BM/B	DNA methylation was associated with poor prognosis.	[80]
C1R	AML	194	B	DNA methylation was associated with the occurrence of specific genomic mutations that are used for risk stratification.	[76]
CDH	MDS	60	BM	DNA methylation was associated with poor prognosis and lower complete remission.	[81]
CDH1	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
CDH1	MDS	37	BM	Hypermethylation can contribute to the development and poor outcome of disease.	[83]
CDH13	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
CDKN2B	MDS	78	BM	DNA methylation was associated with leukemic transformation and disease progression.	[84]
CDKN2B	MDS	25	BM	DNA methylation was associated with pathogenesis and prognosis.	[85]
CEBPA	AML	181	BM	Methylation was associated with better outcome.	[86]
CXXC5	AML	529	BM	Gene was associated with tumor suppressor function in AML and better outcome.	[87]
DLC-1	MDS	43	BM/B	DNA methylation was associated with poor prognosis.	[88]
DLX4	MDS	103	BM	DNA methylation was associated with poor outcome and shorter overall survival	[72]
DNMT3A	LMA	88	B	Methylation was associated with poor prognosis.	[89]
ERalpha-A	Leukemia cases with ERalpha-A methylation (95%; 38 of 40)	40	B	Patients with ERalpha-A methylation had no symptomatic relief and patients without this methylation obtained effective relief. ERalpha-A plays a significant role in leukemogenesis.	[90]
ERalpha-A	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
ERalpha-A	MDS	37	BM	Hypermethylation can contribute to the development and poor outcome of disease.	[83]
EVI1	LMA	476	BM/B	Hipomethylation was associated with poor prognosis.	[91]
EZH2	AML/MDS	54	BM/B	DNA methylation was associated with poor prognosis.	[80]
FHIT	MDS	—	B	DNA methylation was associated with pathogenesis.	[92]
GPX3	MDS	110	BM	DNA methylation was associated with poor prognosis and progression to leukemia in MDS.	[73]
HIC1	MDS	37	BM	Hypermethylation can contribute to the development and poor outcome of disease.	[83]
HIC1	AML	378	BM/B	Hypermethylation was frequently observed in all types of leukemia and strongly correlated with progression to blast crisis.	[93]
HOXA5	AML	378	BM/B	Hypermethylation was frequently observed in all types of leukemia and strongly correlated with progression to blast crisis. Reexpression resulted in the induction of markers of granulocytic differentiation.	[93]
HRK	MDS	60	BM	DNA methylation was associated with advanced stage of MDS and progression.	[94]
ID4	LMA	212	BM	DNA methylation was associated with shorter overall survival	[73]
ID4	MDS	142	BM	DNA methylation was suggested as biomarker for diagnosis.	[95]
ID4	MDS	100	BM	DNA methylation was suggested as biomarker for diagnosis.	[96]
ID4	AML	14	BM	DNA methylation was suggested as biomarker for minimal residual disease detection.	[66]

Table 1: Continued.

Gene	Disease	Patients (n)	Sample type	Associated factors	Ref.
LET-7A-3	MDS	95	BM	DNA methylation was associated with poor prognosis.	[97]
MGMT	AML	21	BM/B	Co-methylation with *p15* gene showed high proportion of leukemic blast cells.	[77]
MGMT	AML	30	BM	DNA methylation was suggested as biomarker to predict therapeutic outcome in male AML patients.	[98]
NOR1	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
NPM2	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
OLIG2	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
p15	AML	21	BM/B	DNA methylation was associated with higher frequency of early death. Comethylation with *MGMT* gene showed high proportion of leukemic blast cells.	[77]
$p15^{INK4b}$	MDS	53	BM	DNA methylation was associated with worse prognosis increasing with disease evolution to AML.	[99]
$p15^{INK4b}$	t-MDS; t-AML	81	BM/B	DNA methylation presented a significantly shorter survival and correlated with loss of chromosome arm 7q.	[100]
$p15^{INK4b}$	MDS	47	BM	DNA methylation was associated with pediatric disease evolution.	[101]
$p15^{INK4b}$	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[83]
$p15^{INK4b}$	MDS	47	BM	DNA methylation was associated with pediatric disease evolution.	[102]
p21	MDS	88	BM	DNA methylation could predict clinical outcome.	[103]
p73	MDS	88	BM	DNA methylation was associated with poor prognosis in de novo MDS.	[103, 104]
PcG	AML	118	BM	DNA methylation was associated with poor prognosis.	[105]
PGRA	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
PGRB	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
PLA2R1	MDS	32	B	DNA methylation was associated with disease evolution in MDS and leukemogenesis	[106]
PLK	Onco-hematological diseases	ND	BM	Promoter methylation correlates with disease and tumorigenesis in blood neoplasms.	[107]
PPARD	AML	344	BM/B	DNA methylation was associated with favorable outcome.	[108]
PSMD2	AML	344	BM/B	DNA methylation was associated with favorable outcome.	[108]
RIL	MDS	317	BM/B	Aberrant DNA methylation predicts overall survival and progression-free survival.	[82]
RING1	AML/MDS	54	BM/B	DNA methylation was associated with poor prognosis.	[80]
sFRP1	MDS	144	BM	DNA methylation was associated with worse overall survival and poor prognosis	[74]
sFRP2	AML	72	BM/B	DNA methylation was associated with increased risk of relapse and risk of death, predicting adverse clinical outcome in patients with normal karyotypes.	[109]
sFRP2	MDS	144	BM	DNA methylation was associated with worse overall survival and poor prognosis	[74]
sFRP5	AML	72	BM/B	DNA methylation was associated with increased risk of relapse and risk of death, predicting adverse clinical outcome in patients with normal karyotypes.	[109]
sFRP5	MDS	144	BM	DNA methylation was associated with worse overall survival and poor prognosis	[74]

Table 1: Continued.

Gene	Disease	Patients (n)	Sample type	Associated factors	Ref.
SOCS-1	MDS	100	B	DNA methylation was associated with disease progression and poor survival	[75]
SOX17	MDS	164	BM	DNA methylation was associated with poor prognosis.	[110]
TERTpro/Ex1	AML	43	BM	Hypermethylation was associated with inferior patient survival.	[111]
TERTpro/Ex1	AML/MDS	33	BM/B	DNA methylation was associated with poor prognosis and inferior patient survival.	[111]
VTRNA1–3	MDS	140	BM	DNA methylation was associated with poor outcome.	[112]
XPNPEP	AML	344	BM/B	DNA methylation was associated with unfavorable outcome.	[108]
ZO-1	MDS	ND	BM	DNA methylation was associated with disease progression.	[113]

AML: acute myeloid leukemia; B: peripheral blood; BM: bone marrow; MDS: myelodysplastic syndrome; ND: not declared; t-AML: therapy-related acute myeloid leukemia; t-MDS: therapy-related myelodysplastic syndrome.

is used to treat these diseases, but there is a chance that more than half of patients will develop resistance to these therapies, leading to worse treatment response [125]. The early identification of whether a patient will respond to treatment is still a major obstacle for achieving clinical success. Evaluations of the clinical course, and subsequent follow-ups, are essential for the safety and efficacy of treatment and for disease remission. Therefore, it is of great importance to identify early markers that may predict which patients will be early responders, late responders, or will not respond at all to treatment.

In a study by Shen and collaborators, it was identified that hypermethylation of *p53*, a vastly studied tumor suppressor gene, and *p73*, which participates in the apoptotic response to DNA damage and, therefore, also acts as a tumor suppressor, correlated strongly with sensitivity to alkylating agents in several cancer cell lines. Six of which were blood- or bone marrow-derived, suggesting that a DNA methylation profile may be useful to identify sensitivity to cancer therapy. However, it should be noted that this study was performed in cultured cell lines and not with patient samples, and, therefore, further studies need to be carried out in order to understand the role of these markers during a patient's clinical course [126].

Another study by Shen and colleagues, with 317 MDS patients, demonstrated that *CDH1*; *CHD13*; *ERα*; *NOR* (oxidored-nitro domain-containing protein isoform 1), a gene that encodes two transcripts and acts as a tumor suppressor; *NPM2* (nucleoplasmin 2), involved in chromatin reprogramming; *OLIG2* (oligodentrocyte lineage transcription factor 2), involved in the chromosomal translocation t(14;21)(q11.2;q22) which is associated with T-cell acute lymphoblastic leukemia; *CDNK2B*; *PGRA* (progesterone receptor A), which functions as transcriptional activator or repressor; and *RIL* (PDZ and LIM domain 4), localized in a region frequently deleted in AML and MDS, were methylated in MDS/AML patients. The methylation pattern before treatment was not associated with clinical response to decitabine. However, methylation reduction after more than four months of treatment correlated with clinical response in 34 patients [127]. In spite of these interesting results, it is important to search for markers that indicate the clinical response before treatment begins or in a shorter time of treatment, in order to aid in choosing the most appropriate therapeutic course for each patient. In a clinical study conducted by Tan and collaborators, it was possible to verify an increase in the acetylation of histones H3 and H4 following treatment with 5-azacitidine combined with panobinostat in AML or MDS patients. The importance of this work stems from the fact that this evaluation was performed utilizing peripheral blood mononuclear cells separated by flow cytometry during the first month of treatment, which is a procedure that could be easily reproduced in other centers [128].

As a matter of fact, genes that have already been related to disease-free survival or disease progression could be reevaluated in peripheral blood in order to corroborate previous findings in bone marrow. For example, the cadherin (CDH) family encodes a calcium-dependent cell-cell adhesion protein, whose loss of function can increase cellular proliferation and invasion, contributing to cancer progression. Other genes, such as $p15^{ink4b}$ and other tumor suppressor genes, encode cyclin-dependent kinase inhibitors which contribute to cell growth regulation and controls cell cycle progression. Data suggest that methylation of this gene could allow leukemic cells to escape inhibitory signals from the bone marrow. The methylation patterns of these two gene families have already been related to AML progression in MDS patients, and, therefore, could be investigated in peripheral blood as well in order to verify if these results are corroborated [83, 99]. The discovery and validation methylation markers in peripheral blood can be very helpful in investigating response during treatment.

During follow-up, in addition to the therapeutic response, toxic effects are evaluated in order to guarantee the patient's safety. Recent studies have sought to correlate epigenetic regulation of cytokines with tumor development [129, 130]. Moreover, cytokine evaluation was suggested as biomarkers for assessing toxicity during treatment, since they are raised significantly in inflammatory responses. However, they present a short serum half-life and lack toxicity-specific expression [131]. Wang and colleagues assessed serum inflammatory cytokines weekly for 15 weeks in patients with

non-small-cell lung cancer during concurrent chemoradiation therapy. An increase in serum IL-6 (interleukin 6), a cytokine that plays a role in inflammation and B-cell maturation, was related to pain, fatigue, disturbed sleep, lack of appetite, and sore throat suggesting a role between proinflammatory cytokine and worsening of symptoms in patients undergoing treatment [132]. With regard to leukemia, Tsapogas and collaborators recently reviewed the role of the cytokine Flt3-ligand (Fms-related tyrosine kinase 3 ligand), which stimulates the proliferation, differentiation, and survival of early hematopoietic cells by activating the FLT3 receptor (Fms-related tyrosine kinase 3), in normal and malignant hematopoiesis [133].

Among the adverse events that may occur during treatment, myelosuppression is the main dose-limiting toxicity and is associated with morbidity and mortality [134, 135]. Febrile neutropenia, or the onset of an infection during neutropenia, represents an emergency and requires administration of broad spectrum antibiotics. These complications may result in reduced dose or interruption of chemotherapy, which compromises patient recovery [101, 136–139]. Moreover, these complications generate high costs, including hospitalization, and may lead to death, demonstrating the importance of its prevention [140, 141].

The understanding of the patient's clinical course, treatment, and risk factors for severe adverse events, such as febrile neutropenia, may allow for preventive actions that reduce the incidence of serious treatment-related complications, all the while reducing the coast of health care [101]. Currently, there are no studies that directly relate and validate changes in epigenetic patterns with the development of toxicity to treatment in hematological malignancies. DNA methylation has already been associated with susceptibility to isoproterenol-induced cardiac pathology in mice. The basal state of the cardiac DNA methylome before and after isoproterenol treatment was compared, and a single-base resolution DNA methylation measurement revealed that treatment decreases global methylation, an event that was associated with heart failure. However, further studies are necessary to investigate this association [142].

Another study with ovarian cancer patients analyzed the methylation in peripheral blood via bisulfite pyrosequencing in different genes during treatment with paclitaxel versus docetaxel. It was observed that higher methylation within the estrogen receptor 1 (*ESR1*) gene was associated with neuropathy on the paclitaxel arm. This was the first cancer study linking DNA methylation in peripheral blood with clinical outcomes, including adverse effects, and suggests that studies evaluating methylation patterns with treatment toxicity in other tumors should also be performed [143]. Another example is the EuroTARGET cohort, a collaborative project that aims to evaluate targeted therapy in renal cell cancer and tumor-related biomarkers for response and toxicity to treatment. Multiplatform "omics," including the methylome, are being employed to identify biomarkers for toxicity; however, the final data is not yet available [144].

With regard to clinical studies, an ongoing study (NCT02259218; clinicaltrials.gov) aims to identify potential biomarkers that may predict the development of radiation pneumonitis in lung cancer patients and radiation necrosis in brain cancer patients. Metabolic and epigenetic profiles are being studied from blood, urine, and tissue samples in order to find biomarkers that are capable of predicting which patients are more likely to develop adverse effects as a result of radiation treatment [145]. Similar studies should be carried out in order to evaluate biomarkers for toxicity before, during, and after treatment in order to predict early toxic events. It is especially important to investigate these biomarkers in peripheral blood, since samples can be obtained with ease and without need of lengthy preparations for the procedure. This would allow for greater patient safety and drug dose adjustment during treatment, optimizing the therapeutic regimen.

4. Conclusion

With regard to MDS and AML, current treatment challenges include choosing the appropriate combination of treatment modalities and chemotherapeutic regimens, since response to therapy is not always achieved. In addition, different adverse effects may occur during treatment because of the toxic effects of most, if not all, chemotherapeutic agents. This seriously delays treatment, affecting the chances of remission, and may directly harm the patient, even leading to death. Moreover, early diagnosis is important in order to increase the potential for a better clinical response during treatment.

Several DNA methylation events affect gene expression and are related to different types of tumors, including hematological malignancies. However, their potential as biomarkers for early diagnosis, stratification, and prediction of treatment response has yet to be more thoroughly evaluated. Studies have demonstrated a significant relationship between DNA methylation patterns and confirmative diagnosis, prognostic potential, and response to treatment. Because changes in DNA methylation are early manifestations and may also act as potential therapeutic targets, the identification of these patterns becomes essential for clinical success. Thus, it is necessary to undertake more studies involving patient samples in order to discover and validate new biomarkers in this field. It is suggested that studies should investigate DNA methylation patterns in peripheral blood samples, in order to optimize not only early diagnosis but also patient management during treatment, allowing for close monitoring of disease progression, adverse events, and response to treatment without the need for bone marrow collection.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors hereby declare that they have no conflict of interest.

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References

[1] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *Cell*, vol. 144, no. 5, pp. 646–674, 2011.

[2] S. B. Baylin and P. A. Jones, "A decade of exploring the cancer epigenome—biological and translational implications," *Nature Reviews Cancer*, vol. 11, no. 10, pp. 726–734, 2011.

[3] J. Sandoval and M. Esteller, "Cancer epigenomics: beyond genomics," *Current Opinion in Genetics & Development*, vol. 22, no. 1, pp. 50–55, 2012.

[4] S. Sharma, T. K. Kelly, and P. A. Jones, "Epigenetics in cancer," *Carcinogenesis*, vol. 31, no. 1, pp. 27–36, 2010.

[5] J. S. You and P. A. Jones, "Cancer genetics and epigenetics: two sides of the same coin?" *Cancer Cell*, vol. 22, no. 1, pp. 9–20, 2012.

[6] A. Hermann, R. Goyal, and A. Jeltsch, "The Dnmt1 DNA-(cytosine-C5)-methyltransferase methylates DNA processively with high preference for hemimethylated target sites," *Journal of Biological Chemistry*, vol. 279, no. 46, pp. 48350–48359, 2004.

[7] L. D. Moore, T. Le, and G. Fan, "DNA methylation and its basic function," *Neuropsychopharmacology*, vol. 38, no. 1, p. 23, 2013.

[8] M. Okano, D. W. Bell, D. A. Haber, and E. Li, "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development," *Cell*, vol. 99, no. 3, pp. 247–257, 1999.

[9] M. A. Mcdevitt, "Clinical applications of epigenetic markers and epigenetic profiling in myeloid malignancies," *Seminars in Oncology*, vol. 39, no. 1, pp. 109–122, 2012.

[10] M. Hatziapostolou and D. Iliopoulos, "Epigenetic aberrations during oncogenesis," *Cellular and Molecular Life Sciences*, vol. 68, no. 10, pp. 1681–1702, 2011.

[11] G. Marcucci, K. Maharry, Y. Z. Wu et al., "IDH1 and IDH2 gene mutations identify novel molecular subsets within de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study," *Journal of Clinical Oncology*, vol. 28, no. 14, pp. 2348–2355, 2010.

[12] S. Abbas, S. Lugthart, F. G. Kavelaars et al., "Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value," *Blood*, vol. 116, no. 12, pp. 2122–2126, 2010.

[13] M. Janin, E. Mylonas, V. Saada et al., "Serum 2-hydroxyglutarate production in IDH1- and IDH2-mutated de novo acute myeloid leukemia: a study by the Acute Leukemia French Association group," *Journal of Clinical Oncology*, vol. 32, no. 4, pp. 297–305, 2014.

[14] K. Lund, P. D. Adams, and M. Copland, "EZH2 in normal and malignant hematopoiesis," *Leukemia*, vol. 28, no. 1, pp. 44–49, 2014.

[15] Z. Li, X. Cai, C. L. Cai et al., "Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies," *Blood*, vol. 118, no. 17, pp. 4509–4518, 2011.

[16] I. Jeziskova, M. Musilova, M. Culen et al., "Distribution of mutations in DNMT3A gene and the suitability of mutations in R882 codon for MRD monitoring in patients with AML," *International Journal of Hematology*, vol. 102, no. 5, pp. 553–557, 2015.

[17] T. J. Ley, L. Ding, M. J. Walter et al., "DNMT3A mutations in acute myeloid leukemia," *New England Journal of Medicine*, vol. 363, no. 25, pp. 2424–2433, 2010.

[18] Cancer Genome Atlas Research Network, "Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia," *The New England Journal of Medicine*, vol. 368, no. 22, pp. 2059–2074, 2013.

[19] M. J. Walter, L. Ding, D. Shen et al., "Recurrent DNMT3A mutations in patients with myelodysplastic syndromes," *Leukemia*, vol. 25, no. 7, pp. 1153–1158, 2011.

[20] H. A. Hou and H. F. Tien, "Mutations in epigenetic modifiers in acute myeloid leukemia and their clinical utility," *Expert Review of Hematology*, vol. 9, no. 5, pp. 447–469, 2016.

[21] D. H. Spencer, D. A. Russler-Germain, S. Ketkar et al., "CpG island hypermethylation mediated by DNMT3A is a consequence of AML progression," *Cell*, vol. 168, no. 5, pp. 801–816, 2017.

[22] Z. K. Otrock, R. V. Tiu, J. P. Maciejewski, and M. A. Sekeres, "The need for additional genetic markers for myelodysplastic syndrome stratification: what does the future hold for prognostication?," *Expert Review of Hematology*, vol. 6, no. 1, pp. 59–68, 2013.

[23] B. Faltas, A. M. Zeidan, and U. Gergis, "Myelodysplastic syndromes: towards a risk-adaptive treatment approach," *Expert Review of Hematology*, vol. 5, no. 5, pp. 611–624, 2013.

[24] O. Salim, T. Toptas, E. Avsar et al., "Azacitidine versus decitabine in patients with refractory anemia with excess blast - results of multicenter study," *Leukemia Research*, vol. 45, pp. 82–89, 2016.

[25] Y. Jing, X. Jin, L. Wang et al., "Decitabine-based chemotherapy followed by haploidentical lymphocyte infusion improves the effectiveness in elderly patients with acute myeloid leukemia," *Oncotarget*, vol. 5, 2016.

[26] A. M. Zeidan, Y. Linhares, and S. D. Gore, "Current therapy of myelodysplastic syndromes," *Blood Reviews*, vol. 27, no. 5, pp. 243–259, 2013.

[27] G. J. Mufti and V. Potter, "Myelodysplastic syndromes: who and when in the course of disease to transplant," *ASH Education Program Book*, vol. 2012, no. 1, pp. 49–55, 2012.

[28] M. Lübbert, S. Suciu, A. Hagemeijer et al., "Decitabine improves progression-free survival in older high-risk MDS patients with multiple autosomal monosomies: results of a subgroup analysis of the randomized phase III study 06011 of the EORTC Leukemia Cooperative Group and German MDS Study Group," *Annals of Hematology*, vol. 95, no. 2, pp. 191–199, 2016.

[29] E. A. Griffiths and S. D. Gore, "Epigenetic therapies in MDS and AML," *Advances in Experimental Medicine and Biology*, vol. 754, pp. 253–283, 2013.

[30] J. P. J. Issa, G. Garcia-Manero, F. J. Giles et al., "Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies," *Blood*, vol. 103, no. 5, pp. 1635–1640, 2004.

[31] X. Luo, H. Wu, Y. Ding, Y. H. Chen, and A. B. Liang, "Clinical efficacy comparison of ultralow dose of decitabine and cyclosporine on low-risk and intermediate-risk type 1 of myelodysplastic syndrome," *Journal of Experimental Hematology*, vol. 24, no. 2, pp. 510–514, 2016.

[32] B. Telek, L. Rejtő, P. Batár et al., "Drug treatment of acute myelogenous leukaemia. Current options and future perspectives," *Orvosi Hetilap*, vol. 157, no. 22, pp. 843–848, 2016.

[33] D. G. Gilliland and J. D. Griffin, "The roles of FLT3 in hematopoiesis and leukemia," *Blood*, vol. 100, no. 5, pp. 1532–1542, 2002.

[34] T. Kitamura, N. Watanabe-Okochi, Y. Enomoto et al., "Novel working hypothesis for pathogenesis of hematological malignancies: combination of mutations-induced cellular phenotypes determines the disease (cMIP-DD)," *The Journal of Biochemistry*, vol. 159, no. 1, pp. 17–25, 2015.

[35] J. M. Foran, "New prognostic markers in acute myeloid leukemia: perspective from the clinic," *ASH Education Program Book*, vol. 2010, no. 1, pp. 47–55, 2010.

[36] E. H. Estey, "Acute myeloid leukemia: 2014 update on risk-stratification and management," *American Journal of Hematology*, vol. 89, no. 11, pp. 1063–1081, 2014.

[37] A. Khwaja, M. Bjorkholm, R. E. Gale et al., "Acute myeloid leukaemia," *Nature Reviews Disease Primers*, vol. 10, no. 2, article 16010, 2016.

[38] C. Y. Cheah, L. J. Nastoupil, S. S. Neelapu, S. G. Forbes, Y. Oki, and N. H. Fowler, "Lenalidomide, idelalisib, and rituximab are unacceptably toxic in patients with relapsed/refractory indolent lymphoma," *Blood*, vol. 25, no. 21, pp. 3357–3359, 2015.

[39] M. Ban-Hoefen, R. Burack, L. Sievert, and D. Sahasrabudhe, "Ipilimumab-induced neutropenia in melanoma," *Journal of Investigative Medicine High Impact Case Reports*, vol. 4, no. 3, pp. 1–5, 2016.

[40] A. M. Brunner, D. B. Costa, R. S. Heist et al., "Treatment-related toxicities in a phase II trial of dasatinib in patients with squamous cell carcinoma of the lung," *Journal of Thoracic Oncology*, vol. 8, no. 11, pp. 1434–1437, 2013.

[41] A. Rashidi and R. B. Walter, "Antigen-specific immunotherapy for acute myeloid leukemia: where are we now, and where do we go from here?," *Expert Review of Hematology*, vol. 9, no. 4, pp. 335–350, 2016.

[42] S. Saußele, M. P. Krauß, R. Hehlmann et al., "Impact of comorbidities on overall survival in patients with chronic myeloid leukemia: results of the randomized CML study IV," *Blood*, vol. 126, no. 1, pp. 42–49, 2015.

[43] D. J. Stewart, G. Batist, H. M. Kantarjian, J. P. Bradford, J. H. Schiller, and R. Kurzrock, "The urgent need for clinical research reform to permit faster, less expensive access to new therapies for lethal diseases," *Clinical Cancer Research*, vol. 21, no. 20, pp. 4561–4568, 2015.

[44] P. A. Jones, "Overview of cancer epigenetics," *Seminars in Hematology*, vol. 42, pp. S3–S8, 2005.

[45] S. Khare and M. Verma, "Epigenetics of colon cancer," *Methods in Molecular Biology*, vol. 863, pp. 177–185, 2012.

[46] M. Verma, P. Maruvada, and S. Srivastava, "Epigenetics and cancer," *Critical Reviews in Clinical Laboratory Sciences*, vol. 41, no. 5-6, pp. 585–607, 2004.

[47] M. Verma, B. K. Dunn, S. Ross et al., "Early detection and risk assessment proceedings and recommendations from the workshop on epigenetics in cancer prevention," *Annals of the New York Academy of Sciences*, vol. 983, no. 1, pp. 298–319, 2003.

[48] S. Y. Kim, D. Y. Shin, S. M. Kim, M. Lee, and E. J. Kim, "Aberrant DNA methylation-induced gene inactivation is associated with the diagnosis and/or therapy of T-cell leukemias," *Leukemia Research*, vol. 47, pp. 116–122, 2016.

[49] Y. Zhang, Q. Jiang, X. Kong et al., "Methylation status of the promoter region of the human frizzled 9 gene in acute myeloid leukemia," *Molecular Medicine Reports*, vol. 14, no. 2, pp. 1339–1344, 2016.

[50] M. G. S. Aithal and N. Rajeswari, "Role of Notch signalling pathway in cancer and its association with DNA methylation," *Journal of Genetics*, vol. 3, pp. 667–675, 2013.

[51] N. Dong, L. Shi, D. C. Wang, C. Chen, and X. Wang, "Role of epigenetics in lung cancer heterogeneity and clinical implication," *Seminars in Cell & Developmental Biology*, vol. 64, pp. 18–25, 2017.

[52] M. Verma, "Epigenetic biomarkers in cancer epidemiology," *Methods in Molecular Biology*, vol. 863, pp. 467–480, 2012.

[53] M. Verma, M. J. Khoury, and J. P. A. Ioannidis, "Opportunities and challenges for selected emerging technologies in cancer epidemiology: mitochondrial, epigenomic, metabolomic, and telomerase profiling," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 22, no. 2, pp. 189–200, 2013.

[54] J. W. Vardiman, J. Thiele, D. A. Arber et al., "The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes," *Blood*, vol. 114, no. 5, pp. 937–951, 2009.

[55] C. Florean, M. Schnekenburger, C. Grandjenette, M. Dicato, and M. Diederich, "Epigenomics of leukemia: from mechanisms to therapeutic applications," *Epigenomics*, vol. 3, no. 5, pp. 581–609, 2011.

[56] M. Esteller, "Epigenetic gene silencing in cancer: the DNA hypermethylome," *Human Molecular Genetics*, vol. 16, no. 1, pp. 50–59, 2007.

[57] S. B. Baylin, "DNA methylation and gene silencing in cancer," *Nature Clinical Practice. Oncology*, vol. 2, pp. 4–11, 2005.

[58] T. E. Fandy, J. G. Herman, P. Kerns et al., "Early epigenetic changes and DNA damage do not predict clinical response in an overlapping schedule of 5-azacytidine and entinostat in patients with myeloid malignancies," *Blood*, vol. 114, no. 13, pp. 2764–2773, 2009.

[59] L. D. P. Silva, M. De Lima, H. Kantarjian et al., "Feasibility of allo-SCT after hypomethylating therapy with decitabine for myelodysplastic syndrome," *Bone Marrow Transplantation*, vol. 43, no. 11, pp. 839–843, 2009.

[60] N. J. Achille, M. Othus, K. Phelan et al., "Association between early promoter-specific DNA methylation changes and outcome in older acute myeloid leukemia patients," *Leukemia Research*, vol. 42, pp. 68–74, 2016.

[61] Z. Liu, J. Zhang, Y. Gao et al., "Large-scale characterization of DNA methylation changes in human gastric carcinomas with and without metastasis," *Clinical Cancer Research*, vol. 20, no. 17, pp. 4598–4612, 2014.

[62] M. J. Fackler, Z. L. Bujanda, C. Umbricht et al., "Novel methylated biomarkers and a robust assay to detect circulating tumor DNA in metastatic breast cancer," *Cancer Research*, vol. 74, no. 8, pp. 2160–2170, 2014.

[63] A. Schröck, A. Leisse, L. de Vos et al., "Free-circulating methylated DNA in blood for diagnosis, staging, prognosis, and monitoring of head and neck squamous cell carcinoma patients: an observational prospective cohort study," *Clinical Chemistry*, vol. 63, no. 7, pp. 1288–1296, 2017.

[64] Y. Li, L. Yang, Y. Pan, J. Yang, Y. Shang, and J. Luo, "Methylation and decreased expression of SHP-1 are related to disease progression in chronic myelogenous leukemia," *Oncology Reports*, vol. 31, no. 5, pp. 2438–2446, 2014.

[65] J. S. Tsang, S. Vencken, O. Sharaf et al., "Global DNA methylation is altered by neoadjuvant chemoradiotherapy in rectal cancer and may predict response to treatment–a pilot study," *European Journal of Surgical Oncology*, vol. 40, no. 11, pp. 1459–1466, 2014.

[66] Y. Zhao, L. Yu, Q. S. Wang et al., "Id4 gene methylation for detection of minimal residual disease in acute leukemia," *Zhonghua Xue Ye Xue Za Zhi*, vol. 27, no. 5, pp. 298–301, 2006.

[67] C. Cobaleda, "Reprogramming of B cells. Cellular programming and reprogramming," *Methods and Protocols*, vol. 636, pp. 233–250, 2010.

[68] M. Park, K. N. Koh, B. E. Kim et al., "Lineage switch at relapse of childhood acute leukemia: a report of four cases," *Journal of Korean Medical Science*, vol. 26, no. 6, pp. 829–831, 2011.

[69] E. Dorantes-Acosta and R. Pelayo, "Lineage switching in acute leukemias: a consequence of stem cell plasticity?," *Bone Marrow Research*, vol. 2012, Article ID 406796, 18 pages, 2012.

[70] E. V. Rothenberg, "T cell lineage commitment: identity and renunciation," *Journal of Immunology*, vol. 186, no. 12, pp. 6649–6655, 2011.

[71] R. Kanwal and S. Gupta, "Epigenetic modifications in cancer," *Clinical Genetics*, vol. 81, no. 4, pp. 303–311, 2012.

[72] T. J. Zhang, J. D. Zhou, D. Q. Yang et al., "Hypermethylation of DLX4 predicts poor clinical outcome in patients with myelodysplastic syndrome," *Clinical Chemistry and Laboratory Medicine*, vol. 54, no. 5, pp. 865–871, 2016.

[73] J. D. Zhou, J. Lin, T. J. Zhang et al., "GPX3 methylation in bone marrow predicts adverse prognosis and leukemia transformation in myelodysplastic syndrome," *Cancer Medicine*, vol. 6, no. 1, pp. 267–274, 2017.

[74] H. Wang, R. Fan, X. Q. Wang et al., "Methylation of Wnt antagonist genes: a useful prognostic marker for myelodysplastic syndrome," *Annals of Hematology*, vol. 92, no. 2, pp. 199–209, 2013.

[75] R. Chaubey, S. Sazawal, M. Mahapatra, S. Chhikara, and R. Saxena, "Prognostic relevance of aberrant SOCS-1 gene promoter methylation in myelodysplastic syndromes patients," *International Journal of Laboratory Hematology*, vol. 37, no. 2, pp. 265–271, 2015.

[76] T. Božić, Q. Lin, J. Frobel et al., "DNA-methylation in C1R is a prognostic biomarker for acute myeloid leukemia," *Clinical Epigenetics*, vol. 4, pp. 7–116, 2015.

[77] K. N. Kurtović, M. Krajnović, A. Bogdanović et al., "Concomitant aberrant methylation of p15 and MGMT genes in acute myeloid leukemia: association with a particular immunophenotype of blast cells," *Medical Oncology*, vol. 29, no. 5, pp. 3547–3556, 2012.

[78] J. Roman-Gomez, A. Jimenez-Velasco, X. Agirre, F. Prosper, A. Heiniger, and A. Torres, "Lack of CpG island methylator phenotype defines a clinical subtype of T-cell acute lymphoblastic leukemia associated with good prognosis," *Journal of Clinical Oncology*, vol. 23, no. 28, pp. 7043–7049, 2005.

[79] A. Guillaumet-Adkins, J. Richter, M. D. Odero et al., "Hypermethylation of the alternative AWT1 promoter in hematological malignancies is a highly specific marker for acute myeloid leukemias despite high expression levels," *Journal of Hematology & Oncology*, vol. 7, no. 1, p. 4, 2014.

[80] F. Xu, X. Li, L. Wu et al., "Overexpression of the EZH2, RING1 and BMI1 genes is common in myelodysplastic syndromes: relation to adverse epigenetic alteration and poor prognostic scoring," *Annals of Hematology*, vol. 90, no. 6, pp. 643–653, 2011.

[81] M. Grövdal, R. Khan, A. Aggerholm et al., "Negative effect of DNA hypermethylation on the outcome of intensive chemotherapy in older patients with high-risk myelodysplastic syndromes and acute myeloid leukemia following myelodysplastic syndrome," *Clinical Cancer Research*, vol. 13, no. 23, pp. 7107–7112, 2007.

[82] L. Shen, H. Kantarjian, Y. Guo et al., "DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes," *Journal of Clinical Oncology*, vol. 28, no. 4, pp. 605–613, 2010.

[83] A. Aggerholm, M. S. Holm, P. Guldberg, L. H. Olesen, and P. Hokland, "Promoter hypermethylation of p15INK4B, HIC1, CDH1, and ER is frequent in myelodysplastic syndrome and predicts poor prognosis in early-stage patients," *European Journal of Haematology*, vol. 76, no. 1, pp. 23–32, 2006.

[84] H. Cechova, P. Lassuthova, L. Novakova et al., "Monitoring of methylation changes in 9p21 region in patients with myelodysplastic syndromes and acute myeloid leukemia," *Neoplasma*, vol. 59, no. 2, pp. 168–174, 2011.

[85] M. Kim, H. Kook, H. J. Park et al., "Quantitative comparison of CDKN2B methylation in pediatric and adult myelodysplastic syndromes," *Acta Haematologica*, vol. 130, no. 2, pp. 115–121, 2013.

[86] T. C. Lin, S. S. Jiang, W. C. Chou et al., "Rapid assessment of the heterogeneous methylation status of CEBPA in patients with acute myeloid leukemia by using high-resolution melting profile," *The Journal of Molecular Diagnostics*, vol. 13, no. 5, pp. 514–519, 2011.

[87] A. Kühnl, P. J. Valk, M. A. Sanders et al., "Downregulation of the Wnt inhibitor CXXC5 predicts a better prognosis in acute myeloid leukemia," *Blood*, vol. 125, no. 19, pp. 2985–2994, 2015.

[88] H. Y. Fu, H. R. Zhou, J. G. Yan, C. J. Chen, and J. Z. Shen, "Clinical significance of hypermethylation of DLC-1 gene in myelodysplastic syndrome patients and effects of decitabine on DLC-1 gene expression," *Zhonghua Yi Xue Za Zhi*, vol. 97, no. 6, pp. 412–417, 2017.

[89] E. Jost, Q. Lin, C. I. Weidner et al., "Epimutations mimic genomic mutations of DNMT3A in acute myeloid leukemia," *Leukemia*, vol. 28, no. 6, pp. 1227–1234, 2014.

[90] J. Yao, X. B. Zhang, X. L. Zhang, and W. L. Fu, "Methylation status of oestrogen receptor α-A: a predictor of prognosis in leukaemias," *Bioscience Reports*, vol. 30, no. 4, pp. 217–222, 2010.

[91] I. Vazquez, M. Maicas, J. Cervera et al., "Downregulation of EVI1 is associated with epigenetic alterations and good prognosis in patients with acute myeloid leukemia," *Haematologica*, vol. 96, no. 10, pp. 1448–1456, 2011.

[92] Y. F. Deng, L. Zhang, X. Q. Zhang, M. Q. Hu, X. Z. Zhang, and Y. L. Xu, "Methylation of FHIT gene promoter region in DNA from plasma of patients with myelodysplastic syndromes and demethylating effect of decitabine," *Zhongguo Shi Yan Xue Ye Xue Za Zhi*, vol. 20, no. 5, pp. 1144–1148, 2012.

[93] G. Strathdee, T. L. Holyoake, A. Sim et al., "Inactivation of HOXA genes by hypermethylation in myeloid and lymphoid malignancy is frequent and associated with poor prognosis," *Clinical Cancer Research*, vol. 13, no. 17, pp. 5048–5055, 2007.

[94] F. Zaker, N. Amirizadeh, N. Nasiri et al., "Gene expression and methylation pattern in HRK apoptotic gene in myelodysplastic syndrome," *International Journal of Molecular and Cellular Medicine*, vol. 5, no. 2, pp. 90–99, 2016.

[95] H. Y. Kang, X. R. Wang, L. Gao et al., "Clinical significance of ID4 gene mehtylation in demethylation-treated MDS cell line and 2 MDS patients," *Zhongguo Shi Yan Xue Ye Xue Za Zhi*, vol. 23, no. 2, pp. 455–459, 2015.

[96] M. Y. Li, Y. Y. Xu, H. Y. Kang et al., "Quantitative detection of ID4 gene aberrant methylation in the differentiation of myelodysplastic syndrome from aplastic anemia," *Chinese Medical Journal*, vol. 128, no. 15, pp. 2019–2025, 2015.

[97] D. H. Wu, D. M. Yao, L. Yang et al., "Hypomethylation of let-7a-3 is associated with poor prognosis in myelodysplastic syndrome," *Leukemia & Lymphoma*, vol. 58, no. 1, pp. 96–103, 2017.

[98] Q. Hong, X. Chen, H. Ye et al., "Association between the methylation status of the MGMT promoter in bone marrow specimens and chemotherapy outcomes of patients with acute myeloid leukemia," *Oncology Letters*, vol. 11, no. 4, pp. 2851–2856, 2016.

[99] B. Quesnel, G. Guillerm, R. Vereecque et al., "Methylation of the p15(INK4b) gene in myelodysplastic syndromes is frequent and acquired during disease progression," *Blood*, vol. 91, no. 8, pp. 2985–2990, 1998.

[100] D. H. Christiansen, M. K. Andersen, and J. Pedersen-Bjergaard, "Methylation of p15INK4B is common, is associated with deletion of genes on chromosome arm 7q and predicts a poor prognosis in therapy-related myelodysplasia and acute myeloid leukemia," *Leukemia*, vol. 17, no. 9, pp. 1813–9, 2003.

[101] G. H. Lyman, M. S. Poniewierski, and E. Culakova, "Risk of chemotherapy-induced neutropenic complications when treating patients with non-Hodgkin lymphoma," *Expert Opinion on Drug Safety*, vol. 15, no. 4, pp. 483–492, 2016.

[102] E. F. Rodrigues, C. B. Santos-Rebouças, M. M. Gonçalves Pimentel et al., "Epigenetic alterations of p15(INK4B) and p16(INK4A) genes in pediatric primary myelodysplastic syndrome," *Leukemia & Lymphoma*, vol. 51, no. 10, pp. 1887–1894, 2010.

[103] Y. Zhao, J. Guo, X. Zhang et al., "Downregulation of p21 in myelodysplastic syndrome is associated with p73 promoter hypermethylation and indicates poor prognosis," *American Journal of Clinical Pathology*, vol. 140, no. 6, pp. 819–827, 2013.

[104] Y. S. Zhao, R. Yang, S. C. Gu et al., "Study of aberrant p73 promoter methylation in patients with myelodysplastic syndrome," *Zhonghua Xue Ye Xue Za Zhi*, vol. 33, no. 10, pp. 847–851, 2012.

[105] S. Deneberg, P. Guardiola, A. Lennartsson et al., "Prognostic DNA methylation patterns in cytogenetically normal acute myeloid leukemia are predefined by stem cell chromatin marks," *Blood*, vol. 118, no. 20, pp. 5573–5582, 2011.

[106] M. Menschikowski, U. Platzbecker, A. Hagelgans et al., "Aberrant methylation of the M-type phospholipase A 2 receptor gene in leukemic cells," *BMC Cancer*, vol. 12, no. 1, p. 576, 2012.

[107] A. Ward, G. Sivakumar, S. Kanjeekal et al., "The deregulated promoter methylation of the polo-like kinases as a potential biomarker in hematological malignancies," *Leukemia & Lymphoma*, vol. 56, no. 7, pp. 2123–2133, 2015.

[108] E. Taskesen, F. J. Staal, and M. J. Reinders, "An integrated approach of gene expression and DNA-methylation profiles of WNT signaling genes uncovers novel prognostic markers in acute myeloid leukemia," *BMC Bioinformatics*, vol. 16, no. 4, article S4, 2015.

[109] E. A. Griffiths, S. D. Gore, C. Hooker et al., "Acute myeloid leukemia is characterized by Wnt pathway inhibitor promoter hypermethylation," *Leukemia & Lymphoma*, vol. 51, no. 9, pp. 1711–1719, 2010.

[110] R. Fan, X. L. Zhao, H. Wang et al., "Abnormal methylation of the sex-determining region Y-box 17 (SOX17) promoter predicts poor prognosis in myelodysplastic syndrome," *Clinical Laboratory*, vol. 60, no. 9, pp. 1465–1474, 2013.

[111] X. Zhao, X. Tian, S. Kajigaya et al., "Epigenetic landscape of the TERT promoter: a potential biomarker for high risk AML/MDS," *British Journal of Haematology*, vol. 175, no. 3, pp. 427–439, 2016.

[112] A. S. Helbo, M. Treppendahl, D. Aslan et al., "Hypermethylation of the VTRNA1-3 promoter is associated with poor outcome in lower risk myelodysplastic syndrome patients," *Genes*, vol. 6, no. 4, pp. 977–990, 2015.

[113] H. Y. Kang et al.X. R. Wang, L. Gao, W. Wang et al., "Clinical implications on ZO-1 gene methylation in myelodysplastic syndrome progression," *Zhongguo Shi Yan Xue Ye Xue Za Zhi*, vol. 23, no. 3, pp. 746–749, 2015.

[114] M. Verma, "The role of epigenomics in the study of cancer biomarkers and in the development of diagnostic tools," *Advances in Experimental Medicine and Biology*, vol. 867, pp. 59–80, 2015.

[115] N. C. Wong, D. Ashley, Z. Chatterton et al., "A distinct DNA methylation signature defines pediatric pre-B cell acute lymphoblastic leukemia," *Epigenetics*, vol. 7, no. 6, pp. 535–541, 2012.

[116] M. Esteller, M. Toyota, M. Sanchez-Cespedes et al., "Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis," *Cancer Research*, vol. 60, no. 9, pp. 2368–2371, 2000.

[117] S. J. Clark, A. Statham, C. Stirzaker, P. L. Molloy, and M. Frommer, "DNA methylation: bisulphite modification and analysis," *Nature Protocols*, vol. 1, no. 5, pp. 2353–2364, 2006.

[118] M. Begemann, I. Leisten, L. Soellner, K. Zerres, T. Eggermann, and S. Spengler, "Use of multilocus methylation-specific single nucleotide primer extension (MS-SNuPE) technology in diagnostic testing for human imprinted loci," *Epigenetics*, vol. 7, no. 5, pp. 473–481, 2012.

[119] X. Calvo, M. Nomdedeu, A. Navarro et al., "High levels of global DNA methylation are an independent adverse prognostic factor in a series of 90 patients with de novo myelodysplastic syndrome," *Leukemia Research*, vol. 38, no. 8, pp. 874–881, 2014.

[120] J. G. Herman, J. Jen, A. Merlo, and S. B. Baylin, "Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B," *Cancer Research*, vol. 56, no. 4, pp. 722–727, 1996.

[121] Y. Oki and J. P. J. Issa, "Epigenetic mechanisms in AML–a target for therapy," *Cancer Treatment and Research*, vol. 145, pp. 19–40, 2010.

[122] I. H. I. Wong, M. H. Ng, D. P. Huang, and J. C. Lee, "Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications," *Blood*, vol. 95, no. 6, pp. 1942–1949, 2000.

[123] N. I. Khan and L. Bendall, "Role of WNT signaling in normal and malignant hematopoiesis," *Histology and Histopathology*, vol. 21, pp. 761–774, 2006.

[124] J. D. Zhou, T. J. Zhang, X. X. Li et al., "Epigenetic dysregulation of ID4 predicts disease progression and treatment outcome in myeloid malignancies," *Journal of Cellular and Molecular Medicine*, vol. 21, no. 8, pp. 1468–1481, 2017.

[125] J. P. J. Issa, "DNA methylation as a therapeutic target in cancer," *Clinical Cancer Research*, vol. 13, no. 6, pp. 1634–1637, 2007.

[126] L. Shen, Y. Kondo, S. Ahmed et al., "Drug sensitivity prediction by CpG island methylation profile in the NCI-60 cancer cell line panel," *Cancer Research*, vol. 67, no. 23, pp. 11335–11343, 2007.

[127] L. Shen, H. Kantarjian, Y. Guo et al., "DNA methylation predicts survival and response to therapy in patients with myelodysplastic syndromes," *Journal of Clinical Oncology*, vol. 28, no. 4, pp. 605–613, 2009.

[128] P. Tan, A. Wei, S. Mithraprabhu et al., "Dual epigenetic targeting with panobinostat and azacitidine in acute myeloid leukemia and high-risk myelodysplastic syndrome," *Blood Cancer Journal*, vol. 4, no. 1, p. 170, 2014.

[129] R. Yasmin, S. Siraj, A. Hassan, A. R. Khan, R. Abbasi, and N. Ahmad, "Epigenetic regulation of inflammatory cytokines and associated genes in human malignancies," *Mediators of Inflammation*, vol. 2015, Article ID 201703, 8 pages, 2015.

[130] B. T. Joyce, T. Gao, L. Liu et al., "Longitudinal study of DNA methylation of inflammatory genes and cancer risk," *Cancer Epidemiology, Biomarkers & Prevention*, vol. 24, no. 10, pp. 1531–1538, 2015.

[131] J. M. Tarrant, "Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use," *Toxicological Sciences*, vol. 117, no. 1, pp. 4–16, 2010.

[132] X. S. Wang, Q. Shi, L. A. Williams et al., "Inflammatory cytokines are associated with the development of symptom burden in patients with NSCLC undergoing concurrent chemoradiation therapy," *Brain, Behavior, and Immunity*, vol. 24, no. 6, pp. 968–974, 2010.

[133] P. Tsapogas, C. J. Mooney, G. Brown, and A. Rolink, "The cytokine Flt3-ligand in normal and malignant hematopoiesis," *International Journal of Molecular Sciences*, vol. 18, no. 6, 2017.

[134] N. M. Kuderer, D. C. Dale, J. Crawford, L. E. Cosler, and G. H. Lyman, "Mortality, morbidity, and cost associated with febrile neutropenia in adult cancer patients," *Cancer*, vol. 106, no. 10, pp. 2258–2266, 2006.

[135] M. A. Lewis, A. W. Hendrickson, and T. J. Moynihan, "Oncologic emergencies: pathophysiology, presentation, diagnosis, and treatment," *CA: a Cancer Journal for Clinicians*, vol. 61, no. 5, pp. 287–314, 2011.

[136] G. H. Lyman and D. J. Delgado, "Risk and timing of hospitalization for febrile neutropenia in patients receiving CHOP, CHOP-R, or CNOP chemotherapy for intermediate-grade non-Hodgkin lymphoma," *Cancer*, vol. 98, no. 11, pp. 2402–2409, 2003.

[137] G. H. Lyman, D. C. Dale, J. Friedberg, J. Crawford, and R. I. Fisher, "Incidence and predictors of low chemotherapy dose-intensity in aggressive non-Hodgkin's lymphoma: a nationwide study," *Journal of Clinical Oncology*, vol. 22, no. 21, pp. 4302–4311, 2004.

[138] G. H. Lyman, "Impact of chemotherapy dose intensity on cancer patient outcomes," *Journal of the National Comprehensive Cancer Network*, vol. 7, no. 1, pp. 99–108, 2009.

[139] M. Shayne, E. Culakova, M. S. Poniewierski et al., "Dose intensity and hematologic toxicity in older cancer patients receiving systemic chemotherapy," *Cancer*, vol. 110, no. 7, pp. 1611–1620, 2007.

[140] J. A. Klastersky and M. Paesmans, "Treatment of febrile neutropenia is expensive: prevention is the answer," *Oncology Research and Treatment*, vol. 34, no. 5, pp. 226–228, 2011.

[141] A. M. Hendricks, E. T. Loggers, and J. A. Talcott, "Costs of home versus inpatient treatment for fever and neutropenia: analysis of a multicenter randomized trial," *Journal of Clinical Oncology*, vol. 29, no. 30, pp. 3984–3989, 2011.

[142] H. Chen, L. D. Orozco, J. Wang et al., "DNA methylation indicates susceptibility to isoproterenol-induced cardiac pathology and is associated with chromatin states," *Circulation Research*, vol. 118, no. 5, pp. 786–797, 2016.

[143] J. M. Flanagan, C. S. Wilhelm-Benartzi, M. Metcalf, S. B. Kaye, and R. Brown, "Association of somatic DNA methylation variability with progression-free survival and toxicity in ovarian cancer patients," *Annals of Oncology*, vol. 24, no. 11, pp. 2813–2818, 2013.

[144] L. F. van der Zanden, S. H. Vermeulen, A. Oskarsdottir et al., "Description of the EuroTARGET cohort: a European collaborative project on TArgeted therapy in renal cell cancer-GEnetic- and tumor-related biomarkers for response and toxicity," *Urologic Oncology*, vol. 35, no. 8, 2017.

[145] A. Chakravarti, "Longitudinal metabolomic and epigenetic profiling of bodyfluids from patients with lung and brain cancer receiving radiation therapy," 2014, https://www.clinicaltrials.gov/ct2/show/NCT02259218.

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Review Article

DNA Methylation as a Noninvasive Epigenetic Biomarker for the Detection of Cancer

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In light of the high incidence and mortality rates of cancer, early and accurate diagnosis is an important priority for assigning optimal treatment for each individual with suspected illness. Biomarkers are crucial in the screening of patients with a high risk of developing cancer, diagnosing patients with suspicious tumours at the earliest possible stage, establishing an accurate prognosis, and predicting and monitoring the response to specific therapies. Epigenetic alterations are innovative biomarkers for cancer, due to their stability, frequency, and noninvasive accessibility in bodily fluids. Epigenetic modifications are also reversible and potentially useful as therapeutic targets. Despite this, there is still a lack of accurate biomarkers for the conclusive diagnosis of most cancer types; thus, there is a strong need for continued investigation to expand this area of research. In this review, we summarise current knowledge on methylated DNA and its implications in cancer to explore its potential as an epigenetic biomarker to be translated for clinical application. We propose that the identification of biomarkers with higher accuracy and more effective detection methods will enable improved clinical management of patients and the intervention at early-stage disease.

1. Types of Noninvasive Epigenetic Biomarkers

1.1. The Need for Biomarkers in the Diagnosis of Cancers. Novel diagnostic and prognostic biomarkers are urgently needed to aid in the prevention and management of cancers worldwide. The detection of aberrantly expressed biological molecules manifested during carcinogenesis can serve as a guideline for clinicians to make informed judgments based on predicted variables such as the likelihood of metastasis, tumour recurrence, and expected length of patient survival [1]. Currently, there is a shortage of noninvasive biomarkers with sufficient accuracy to identify patients in need of treatment, especially during the early stages of cancer where intervention has the highest potential to reduce mortalities [2]. Unfortunately, cancer diagnosis is complex and is often confounded by issues such as the long latency periods of some tumours and lack of clinical presentation at the early stages of disease [1, 3]. As a result of this, delayed intervention is a frequent occurrence, which facilitates the progression into more advanced stages of cancer. Therefore, the discovery and development of novel biomarkers are urgently needed for the screening of high-risk populations to enable prompt and successful treatment [1].

1.2. Limitations of Current Detection Methods for Cancer. Currently, there are numerous noninvasive techniques for the detection of specific cancer types including computed tomography (CT) in lung cancer, mammography in breast cancer, or positron emission tomography (PET) scan and digital rectal examination (DRE) for prostate cancer [4–6]. Many imaging techniques have a high sensitivity for the detection of abnormal neoplasms but lack the capacity to

classify tumour subtypes or distinguish benign from malignant tumours [7]. In breast cancer screening, the use of mammography is limited to the detection of larger tumours, resulting in the neglect of smaller tumours [8], and diagnosis is dependent on the level of expertise possessed by the clinician [6]. In prostate cancer, as part of the physical examination of the prostate gland, the DRE is semi-invasive and can cause injury and bleeding to the patient [4]. The current gold standard for cancer diagnosis is histopathology; this method is dependent on invasive and often painful techniques. Techniques such as fine needle aspiration (FNA) and a core biopsy are required for the extraction of suspected tumour tissue and subsequent histological evaluation [9]. Histopathological assessment has its limitations, and the accuracy of results is dependent on the quality and yield of tissue obtained. For example, only 0.03% of tissue from the entire prostate is removed during core needle biopsy and may not accurately represent the actual tumour core when assessing for prostate cancer [10]. There is also an inherent risk of physical damage to functional organs during biopsy, for example, the possibility of pneumothorax during the investigation of respiratory neoplasms [11]. A further limitation of histological analysis is that tissue analysis is subject to the interpretation of the pathologist, which can be unreliable in the case of rare cancers or cancers with ambiguous histological features [12]. Therefore, it is imperative that for the purposes of histopathology, a representative section of tumour status is obtained; however, this cannot be easily guaranteed when retrieving tumour tissue during biopsy [10].

1.3. The Impact of DNA Methylation in Carcinogenesis. The most widely studied epigenetic alteration to date is the methylation (5-methylcytosines) of DNA at the CpG dinucleotides, which are highly concentrated in the CpG islands within the promoter region or near the first exon [13]. Varying degrees of methylation within a gene's CpG islands leads to various levels of gene silencing, and in cancer, promoter hypermethylation has been linked to the silencing of tumour suppressor genes and subsequent oncogenesis [14–16]. Screening for gene mutations is a common practice to test for an individual's predisposition to cancer but cannot reflect the current status or activity of disease [17]. Additionally, promoter methylation is often easier to evaluate due to its defined location within the promoter region of specific genes. Comparatively, locating gene mutations is more complex as they can occur at unsuspected sites within the gene that may be challenging to pinpoint. Some epigenetic markers have value in the early detection of cancers due to their involvement in the initiation of carcinogenic pathways [18, 19]. As a consequence, epigenetic biomarkers have a high potential and wide scope to be implemented as early diagnosis biomarkers.

1.4. Noninvasive Epigenetic Biomarkers for Cancer. Despite the benefits of current noninvasive detection methods for the screening of cancer, the accuracy of results is still limited. To achieve a conclusive and accurate diagnosis, the use of invasive techniques are necessary. Epigenetic biomarkers can be extracted using noninvasive techniques to determine prognosis and accurately predict the outcomes of disease [20]. Recent developments in epigenetics have explored the use of biological fluids including blood-based biomarkers, which are under investigation for their potential to limit the need for biopsy [21]. A good source of tumour-derived nucleic acids is peripheral blood, which can be retrieved noninvasively through venipuncture also referred to as liquid biopsy [22]. Circulating cell-free DNA can be isolated from plasma or serum for the evaluation of epigenetic changes. One of the major areas in epigenetic-based biomarker research is promoter methylation which can be detected in DNA extracted from bodily fluids such as serum, saliva, and urine.

1.5. Types of Noninvasive Biospecimens for the Detection of Methylated DNA. Individual cancers, depending on their anatomical location, have characteristic mechanisms for shedding tumour DNA into closely related bodily fluids. These biological fluids can be exploited as a source for biomarker investigation. For example, urine and urinary sediment can harbor carcinoma cells that are not accessible through biopsy from bladder cancer [23–25] or prostate cancer [10]. Therefore, urine represents an attainable source of tumour-derived DNA that is easily excreted and collected in a noninvasive manner [26–28]. Sputum can possess malignant cells from lung cancer and has been determined to provide a more accurate methylation status compared to blood-based samples [29–32], as reported when observing a salivary rinse for oral cancer [33]. This is a result of the copious amounts of DNA shed from the tumour cells from the thoracic and oral regions into sputum and saliva. Stool can also be used to detect tumour-derived methylated DNA biomarkers for colorectal cancer [34–36] and pancreatic cancer [37]. A large proportion of the sample types evaluated to detect circulating cell-free biomarkers are blood based [38, 39] in the detection of many cancers [40, 41] as it contains a high volume of genetic material. Comparatively, plasma provides a more accurate representation of circulating cell-free DNA for the detection of cancers to serum, which can contain DNA contaminations as a result of coagulation [42].

2. Methods for the Detection of DNA Methylation in Tissue and Biological Fluid

There are numerous methods that can be applied for detection of epigenetic biomarkers, which encompass whole genome screening, pyrosequencing, quantitative methylation-specific PCR (qMSP), MethyLight assay, and one-step methylation-specific polymerase chain reaction (OS-MSP) assay [43–47]. The variety of techniques that have been developed to detect DNA methylation each has its own advantages and limitations [48]. Genome-wide methylation sequencing or microarray-based profiling is often used to identify candidate biomarkers, whereas the performance of a specific marker or a limited panel of markers in larger cohorts is typically assessed using locus-specific assays such as quantitative methylation-specific PCR (qMSP), one-step methylation-

specific PCR, MethyLight assay, and pyrosequencing, which can detect methylation of known loci with high sensitivity and specificity [43–45, 49, 50].

3. Noninvasive Epigenetic Markers in Cancers (Table 1)

3.1. Prostate Cancer. Prostate cancer is amongst the most frequently diagnosed cancers in the world, affecting 31.1 per 100,000 men [1] and accounting for 1 death every 4 minutes [51]. Determining the prognosis of prostate cancer is difficult due to the lack of accuracy in the biomarkers currently available [21]. The developments of noninvasive detection biomarkers for prostate cancer will largely facilitate the management of this cancer. Serum prostate-specific antigen (PSA), the conventional marker used to diagnose prostate cancer, is upregulated in individuals with the disease; however, it has poor sensitivity and specificity as an individual marker [21]. A number of genes with tumour suppressor functions have been assessed for epigenetic changes in prostate cancer to provide an alternative biomarker to PSA. Brait et al. recently used qMSP to test 10 genes previously associated with methylation in prostate cancer tissue and developed a panel of 3 methylated genes to assist in the detection of prostate cancer [21]. In this study, serum samples from 84 prostate cancer patients, 30 cancer-free controls, and 7 patients with the precancerous prostate abnormality high-grade prostatic intraepithelial neoplasia (HGPIN) were evaluated for promoter methylation [21]. Of the 10 genes tested, the methylation status of *SSBP2*, *MCAM*, *ERα*, *ERβ*, *CCND2*, *MGMT*, *GSTP1*, and *p16* genes were best matched when comparing the results from serum to prostate cancer tissue [21]. *MCAM* methylation was most accurate (with AUC of 0.66 obtained from the ROC), being detected in 85% of the early-stage (T1c) cancers ($n = 60$). When combining *MCAM* methylation with PSA threshold of >4 ng/ml [21], there was 91% detection of early-stage cancers. Finally, detection of at least one methylated gene in a panel of 3 genes, *MCAM*, *ERα*, and *ER*, improved specificity to 70% in comparison to serum PSA, which was only 30% [21]. Deng et al. used qMSP and found that hypermethylation in serum promoter *protocadherin 10* (*PCDH10*) was associated with worse prognosis and shorter survival from patients undergoing preporostate transurethral resection [22]. They included 171 prostate cancer patient samples and 65 controls with benign prostatic hyperplasia and found 51.5% of hypermethylation in cancer correlated to preoperative high PSA level ($p = 0.001$) [22], worse prognosis, and lymph node metastasis [22]. Similarly, Wang et al. showed that methylated serum *CDH13* was detected in 44.9% of 98 prostate cancer samples (with shorter survival) and undetectable in 47 control serum [52]. Increased *CDH13* promoter methylation was also associated with higher PSA levels [52]. Both studies indicated no methylation of the *PCDH10* and *CDH13* in benign prostate controls.

The most frequently studied epigenetic marker in prostate cancer is *Glutathione S-transferase 1* (*GSTP1*) [53]. It is methylated in prostate cancer tissue and most prostate cancer cell lines [54]. Recently, *GSTP1* promoter methylation has been evaluated for its potential as a noninvasive epigenetic marker in peripheral blood [41] and urine [10, 53, 55]. *GSTP1* is commonly methylated in prostate cancer tissue, blood, and urine, which has been confirmed in multiple studies [10, 53, 55]. *GSTP1* methylation in plasma was detected using qMSP in a phase I exploratory cohort of 75 men and further validated in an independent cohort of 51 men [41]. *GSTP1* hypermethylation was associated with poor prognosis and poor overall survival and was a good predictor for worse prognosis after treatment with chemotherapy [41].

Daniunaite et al. defined a panel of methylated promoter genes, which included *GSTP1*, *RASSF1*, and *RARB* for the detection of prostate cancer in urine [55]. One or more of the 3 genes were detected using qMSP in 82% of 37 catheter urine samples from patients with prostate cancer [55]. Jatkoe et al. found that hypermethylation of GSTP1 and APC in urine was a highly sensitive biomarker for early diagnosis using a cohort of 665 prostate cancer patients. The methylated gene combination was also more representative of disease status than Gleason score used to analyse biopsy tissue [10]. A study by Woodson et al. evaluated *GSTP1* methylation as an independent biomarker in urine with a 75% sensitivity and 98% specificity rate in urine compared to the 88% specificity and 91% sensitivity of prostate cancer tissue specimens [53]. The detection of *GSTP1* promoter methylation in urine was significantly higher in stage III at 100% when compared to 20% in stage II samples ($p = 0.05$) [53].

3.2. Bladder Cancer. Bladder cancers are a rapidly progressing illness with high prevalence and varied symptoms from patient to patient [56]. Patients with previous disease require persistent screening posttreatment, based on a high risk of tumour recurrence [57]. The current gold standard for diagnosis is cystoscopy which is invasive, and its high cost and complexity render this method inappropriate for repetitive screening [23, 24]. Urine in bladder cancer contains cancer cells exfoliated from the epithelial lining of the bladder containing tumour-derived DNA. Studies have successfully identified urine as a source for detection of epigenetic modifications [23, 24]. Several studies have evaluated methylation of multiple tumour suppressor genes to determine their relationship with bladder cancer tissue and subsequent methylation status in urine, and some of these genes include *APC*, *ARF*, *BAX*, *BCL2*, *CDH1*, *CDKN2A*, *DAPK*, *EDNRB*, *EOMES*, *FADD*, *GDF15*, *GSTP1*, *LITAF*, *MGMT*, *NID2*, *PCDH17*, *POU4F2*, *RARβ2*, *RASSF1A*, *TCF21*, *TERT*, *TIMP3*, *TMS-1*, *TNFRSF21*, *TNFRSF25*, and *ZNF154* [24, 45, 58].

Wang et al. studied the combination of hypermethylated *POU4F2* and *PCDH17* and found 90% sensitivity and 93.6% specificity in a cohort of 312 individuals using qMSP [24] when compared to healthy controls and other pathological bladder conditions including infected urinary calculi, kidney cancer, and prostate cancer [24]. Similarly, a study by Hoque et al. confirmed an increase in *CDKN2A*, *ARF*, *MGMT*, and *GSTP1* methylation status to correlate with tumour progression whilst in healthy control samples, methylation was undetectable when using the qMSP technique [58]. Friedrich et al. studied *DAPK*, *BCL2*, and *TERT* and found that they were methylated in bladder cancer when comparing to 20

Table 1: Noninvasive epigenetic DNA methylation biomarkers used in cancers.

Methylated gene	Marker (Dx/Px)	Cancer	DNA source	Cohort	Method	Accuracy (statistic of individual gene)	Ctrl†	Accuracy of panel including methylated gene		Ref
								Genes (*n*)	Statistic	
ARF	Dx	Bladder	Urine sediment	15 BlCa, 25 NC	qPCR	†27%, (4/15)	100% -ve	4	Δ82%/96%	[58]
APC	Px	Prostate	Urine	665 men with elevated PSA	qPCR	—	—	2	—	[10]
BCL	Dx	Bladder	Urine sediment	37 BlCa, 20 NC	MethyLight	†65% (24/37)	100% -ve	3	†78% (29/37)	[45]
CDH13	Px	Prostate	Serum	98 PCa, 47 NC	qMSP	†45%, 44/98	100% -ve	—	—	[52]
CDKN2A	Dx	Bladder	Urine sediment	15 BlCa, 25 NC	qPCR	†47%, 7/15	100% -ve	4	Δ82%/96%	[58]
DAPK	Dx	Bladder	Urine sediment	37 BlCa, 20 NC	MethyLight	†22% (8/37)	100% -ve	3	†78% (29/37)	[45]
ERα	Dx	Prostate/breast (primary)	Serum	16 PCa/120 BCa, 100 BN	qMSP	†13% (2/16), †72%	—	3	Δ75%/70%	[21, 77]
ERβ	Dx	Prostate	Serum	16 PCa	qMSP	†13% (2/16)	—	3	Δ75%/70%	[21]
FBN1	Dx	Colorectal	Stool	89 CRC, 30 NC	qMSP	†70.8% (63/89	93% -ve	2	Δ84.3%/93.3%	[35]
FBN2	Dx	Colorectal (primary)	Serum	78 CRC	qMSP	†8% (4/49)		—	—	[62]
GSTP1	Dx, Px	Bladder/prostate/castrate-resistant prostate/breast	Urine sediment/urine/plasma/serum	15 BlCa, 25 NC/665 men w/ elevated PSA/34 PCa/24 PCa, 8HGPIN, BN/75 CR-PCa/120 BCa/101 BCa (primary), 58 BCa (secondary), 87 healthy	qPCR, OS-MSP	†47% 7/15/ †4% 4/101/ †3% 1/34/ †67% 50/75	100% -ve	4, 2, 3, 3, 3	Δ82%,96%/−/ †82% (28/34)/ Δ75%/98%/ †6% 7/120/ †22% 22/101	[10, 41, 43, 44, 53, 55, 58]
FHIT	Dx	Ductal breast cancer	Serum	36 BCD, 30NC, 30 BN	qMSP and high-resolution melting curve analysis	64%	35% NC, 64% BC	—	—	[76]

Table 1: Continued.

Methylated gene	Marker (Dx/Px)	Cancer	DNA source	Cohort	Method	Accuracy (statistic of individual gene)	Ctrl†	Accuracy of panel including methylated gene		Ref
								Genes (*n*)	Statistic	
hMLH1	Dx	Breast	Serum	268 BCa, 245 NC, 236 BN	MethyLight	†28% 75/268	85.71% -ve NC, 77.97% -ve BN	6	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)	[8]
HLTF	Px	Colorectal	Serum	106 CRC	MethyLight	—	—	—	—	[20]
HOXD13	Dx	Breast	Serum	268 BCa, 245 NC, 236 BN	MethyLight	†37/268	97.55% -ve NC, 99.58% -ve NC	6	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)	[8]
MCAM	Dx (early)	Prostate	Serum	16 PCa	qMSP	†46% (12/16)	—	3	Δ75%/70%	[21]
MGMT	Dx	Bladder/lung	Urine sediment/ bronchial washing	15 BlCa, 25 NC/8 SCLC	qPCR/MSP	†27% 4/15, †75% 6/8	100% -ve	4	Δ82%/96%	[52, 73]
NID2	Dx	Bladder (primary)	Urine	157 BlCa, 339 urological disorder	qMSP	Δ94%, 91%	—	2	†94% (466/496)	[84]
P16	Dx	Breast	Serum	268 BCa, 245 NC, 236 BN	MethyLight	†22% 60/268	83.27% -ve NC, 84.32% -ve BN	6	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)	[8]
PCDHGB7	Dx	Breast	Serum	268 BCa, 245 NC, 236 BN	MethyLight	†148/268	*52.65% -ve NC, 54.66% -ve BN*	6	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)	[8]

Table 1: Continued.

Methylated gene	Marker (Dx/Px)	Cancer	DNA source	Cohort	Method	Accuracy (statistic of individual gene)	Ctrl†	Accuracy of panel including methylated gene		Ref
								Genes (*n*)	Statistic	
PCDH10	Px	Prostate	Serum	171 primary PCa, 65 BN	qMSP	†51.5% (88/171)	100% -ve	—	—	[22]
PCDH17	Dx	Bladder	Urine sediment	58 BlCa, 20 IUC, 20 KCa, 20 PCa	qMSP	AUC = 0.813	75% -ve	2	Δ90%/93.96%	[24]
PHACTR3	Dx	Colorectal	Stool	11 CRC, 20 NC	qMSP	†44% 4/9	100% -ve	—	—	[85]
POU4F2	Dx	Bladder	Urine sediment	58 BlCa, 20 IUC, 20 KCa, 20 PCa	qMSP	AUC = 0.921	94% -ve	2	Δ90%/93.96%	[24]
TERT	Dx	Bladder	Urine sediment	37 BlCa, 20 NC	MethyLight	†51% (18/37)	100% -ve	3	†78% (29/37)	[45]
TMEFF2	Dx	NSCLC	Serum	316 NSCLC, 50 NC		†9.2% 29/36	100% -ve	—	—	[70]
RARB	Dx	Prostate	Urine sediment	34 PCa	qMSP	†44% (15 of 34)		3	†82%(28/34)	[55]
RARβ2	Dx	Breast	Serum	120 BCa//101 BCa (primary), 58 BCa (secondary), 87 healthy	OS-MSP	–, †12% 12/101	—	3, 3	†6% 7/120/ †22% 22/101	[43, 44]
RASSF1	Dx	Prostate	Urine sediment	34 PCa	qMSP	†71% 24/34		3	†82% (28/34)	[55]
RASSF1a	Dx, Px	Breast/lung/ovarian	Serum	268 BCa, 245 NC, 236 BN/101 BCa (primary), 58 BCa (secondary), 87 healthy /120 BCa/90 LCa/59 HGSC	qMSP/OS-MSP/ MSP, MS-HRMA	†17% 46/268, †7% 7/101 †33.8% 27/80, †25.4% 15/59	89.67% -ve NC, 91.95% -ve BN/ 100% -ve	6, 3	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)/ †6% 7/120/ †22% 22/101	[8, 43, 44]
RASSF2A	Dx	Ovarian	Plasma	47 EOC, 14 BN, 10 NC	MSP	†36.2% 17/47	100% -ve BN, 100% -ve NC	—	—	[82]

Table 1: Continued.

Methylated gene	Marker (Dx/Px)	Cancer	DNA source	Cohort	Method	Accuracy (statistic of individual gene)	Ctrl†	Accuracy of panel including methylated gene		Ref
								Genes (*n*)	Statistic	
SEPT9	Px	Colorectal	Serum	150 CRC, 60 CRC	qMSP, Abbott MS-9 reagent kit and PCR	–, Δ47%/ 89%	—	—	—	[34, 63]
SFN	Dx	Breast	Serum	268 BCa, 245 NC, 236 BN	MethyLight	†74% 197/ 268	85.71%-ve NC, 77.97% -ve BN	6	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)	[8]
SNCA	Dx	Colorectal	Stool	89 CRC, 30 NC	qMSP	†70% 62/89	100% -ve	2	Δ84.3%/93.3%	[35]
SST	Px	Colorectal	Serum	165 CRC	—	—	—	—	—	[59]
TAC1	Px	Colorectal	Serum	150 CRC	qMSP	—	—	—	—	[63]
TWIST1	Dx	Bladder (primary)	Urine	157 BlCa, 339 urological disorder	qMSP	Δ88%, 94%	—	2	†94% (466/496)	[84]
VIM	Dx	Colorectal	Serum	44 CRC, 239 CRC	qMSP	†9% 4/44, †33% 78/239	—	—	—	[61, 65]

Dx: diagnosis; Px: prognosis; BN: benign controls; NC: noncancer controls; Δ: sensitivity/specificity; †: % of samples detected; AUC: area under the curve (0-1) for ROC (receiver operating characteristic); BlCa: bladder cancer; BCa: breast cancer; BCD: breast ductal carcinoma; CR-PCa: castrate resistant prostate cancer; CRC: colorectal cancer; HGSC: high-grade serous ovarian cancer; IUC: infected urinary calculi: KCa: kidney cancer: LCa: lung cancer, NSCLC: non-small-cell lung cancer; PCa: prostate cancer; SCLC: small cell lung cancer.

healthy controls [45]. Hypermethylation of *TERT* and *BCL2* was associated with tumour grading, and *BCL2* was associated with tumour stage [45]. Utilising a microdroplet-based PCR, a high-throughput next-generation sequencing approach targeting DNA methylation, Feber et al. developed a 150 CpG loci test called UroMark [23]. This genome-wide methylation profile detected bladder cancer with high accuracy by receiver operator characteristic (ROC) and achieved 0.97 of area under the curve (AUC) from overall 167 noncancer controls and 107 bladder cancer samples [23].

3.3. Colorectal Cancer. Colorectal cancer has the 3rd highest global prevalence of cancers with 1.9 million men and 1.6 million women affected, and its incidence is more common in developed countries, recorded by WHO in 2014 [1]. Due to the high recurrence rates of colorectal cancer, there is an urgency to develop noninvasive biomarkers appropriate for frequent screening [59]. The current major form of diagnosis for colorectal cancer is through colonoscopy which is an invasive and time-consuming method [60]. Aberrant methylation of genes can be used in such a way that they reflect cancer stage [17] and subsequently exploited to aid clinicians as diagnostic or prognostic biomarkers.

Various genes related to malignant development have been investigated in the tissue and bodily fluids of colorectal cancer patients including *Fibrillin-2* (*FBN2*), *MAL*, septin 9 (*SEPT9*), tachykinin-1 (*TAC1*), nel-like type 1 (*NELL1*), cellular retinoic acid binding protein 1 (*CRABP1*), somatostatin (*SST*), eyes absent homolog 4 (*EYA4*), and *Vimentin* (*VIM*) [59, 61, 62]. The current biomarkers used in the clinic for detection of colorectal cancer are carcinoembryonic antigen (CEA) and CA19-9 which have limited accuracy for independent diagnosis [63]. The following studies have compared the validity of these genes as potential biomarkers in colorectal cancer.

Serum hypermethylation of the *SST* and *MAL* genes were confirmed by Liu et al. in a study including 165 preoperative stages II and III colorectal cancer patients [59]. Hypermethylation was correlated to high recurrence of tumours in a 56-month median follow-up study of serum *SST* and *MAL* [59]. In a similar study, Tham et al. identified increased methylation of *TAC1*, *SEPT9*, and *NELL1* genes in serum to be correlated to poor prognosis in colorectal cancer in a cohort of 150 patients [63]. This study concluded that hypermethylation of *TAC1* and *SEPT9* promoter regions was detectable earlier in patients postresection compared to CEA and was a better predictor of tumour recurrence [63]. Additionally, it was found that the risk of cancer-specific mortality increased with hypermethylation of the *NELL1* promoter region [63]. Herbst et al. investigated the preoperative serum of 106 colorectal cancer patients followed by curative resection and revisited in a 5-year follow-up study [20]. This research found *HLTF* methylation to be significantly higher in patients that experienced tumour recurrence ($p = 0.014$) [20]. Therefore, the detection of methylation in the genes *SST*, *TAC1*, *SEPT9*, and *HLTF* may enable early detection and assist in more effective intervention during postresection tumour recurrence compared to conventional markers. Of these genes, *SEPT9* has recently obtained FDA approval for use as a noninvasive methylation marker in the clinic [64] and is distributed as the commercial test (Epi proColon test).

Silencing of *VIM* by promoter methylation has been linked to poor prognosis in colorectal cancer using qMSP to detect serum methylation level in several studies [61, 65]. *VIM* promoter methylation was tested in conjunction with CEA and carbohydrate antigen (CA 19-9) [65], which are elevated in the more advanced stages of colorectal cancer [66]. In the early stages (0–II) of colorectal cancer, detection of *VIM* methylation had a higher sensitivity than CEA and CA 19-9 [65]. When we combined results from 3 serum markers, the sensitivity was significantly increased to 85.7% for detection of patients with stage IV tumours compared to earlier stage cancers [65]. In a study by Shirahata et al., hypermethylation of *VIM* was detected in only 9% of the 44 serum samples from colorectal cancer patients [61]. However, when assessed in conjunction with clinicopathological features, *VIM* methylation was significantly increased in patients with advanced disease who had developed distant metastasis ($p = 0.0063$), secondary tumours in the liver ($p = 0.026$), and peritoneal dissemination ($p = 0.0029$) [61]. In a further study, the *Fibrillin-2* (*FBN2*) gene was determined to be hypermethylated in colorectal cancer tissue [62]. This was later confirmed in the serum of colorectal cancer patients with hepatic metastasis ($p < 0.0001$) and found at higher frequency in male patients [62]. The fecal occult blood test (FOBT) is used in the conventional testing for the detection of colorectal cancer in which stool is analyzed for the presence of blood from bowel lesions [34]. Chen et al. tested the results from FOBT in combination with *SEPT-9* hypermethylation in plasma [34]. A more accurate diagnosis for colorectal cancer was obtained yielding an AUC of 0.766 when compared to each technique performed individually [34].

Epigenetic changes associated with malignant bowel tissue have been detected in the stool of patients with colorectal cancer, rendering it a source for biomarker detection [35]. Li et al. determined that the presence of hypermethylation in at least one of the genes *SNCA* and *FBN1* was found to have a sensitivity and specificity of 84.3% and 93.3%, respectively [35]. This study examined 89 stool samples from colorectal cancer patients and 30 healthy controls using qMSP [35]. Glockner et al. discovered that tissue factor pathway inhibitor 2 (*TFPI2*) was methylated in 99% of 115 stool samples from colorectal cancer patients [67]. From the early to advance stages, sensitivity increased from 76% to 79% and specificity from 89% to 93%, suggesting the use of methylated *TFPI2* with a good accuracy for screening from the early stages of carcinogenesis [67].

3.4. Lung Cancer. Lung cancer is a commonly diagnosed and aggressive respiratory tumour with poor survival [1]. There are numerous subtypes to the disease with risk factors including age, tobacco smoking, and family history [1]. Initial screening for respiratory lesions is performed by computed tomography (CT), which is highly sensitive for the detection

of lung cancer, but is subject to a high rate of false-positive results from the detection of benign tumours [7, 68]. There are serveral circulating biomarkers under investigation for screening of lung cancers; however, none exist with the accuracy to eliminate biopsy and histopathology for the final diagnosis [69]. Transmembrane protein with a single EGF-like and two follistatin domains (*TMEFF2*) is inactivated through promoter methylation in numerous cancers including non-small-cell lung cancer (NSCLC) [70]. Hypermethylation of *TMEFF2* is more common in non-*EGFR* mutation female patients ($p = 0.06$) and subjects who have never smoked ($p = 0.07$) [70]. In this study, serum samples from 316 NSCLC patients and 50 healthy age-matched controls were used and *TMEFF2* hypermethylation was detectable in 9.2% of NSCLC cases and no methylation in healthy controls [70]. Hypermethylation of *RASSF1A* was detected in 33.8% of NSCLC and not in healthy controls of benign pulmonary disease in the cohort of 80 patients [14].

Sputum as a biospecimen is more representative of NSCLC compared to blood due to the direct shedding of tumour DNA from the lung [14, 71]. Belinsky et al. used sputum and serum to identify methylation targets for NSCLC and found that *p16*, *DAPK*, *PAX5b*, and *GATA5* were potential biomarkers for NSCLC [71]. They included 72 stage III NSCLC patients to compare tumour tissue, sputum, and serum [71]. Methylation status was higher in sputum and similar to tumour tissue but low in serum [71]. Su et al. analysed microRNA and DNA methylation in the cohort of 117 stage one NSCLC patients and 174 healthy smokers. Results indicated methylation of *RASSF1A*, *PRDM1*, and *3OST2* were useful for early detection at stage 1. They also found that miR-21, miR-31, and miR-210 were sensitive for early detection in between 62% and 77% of cases [29]. Even though these genes could facilitate cancer detection at earlier stages, further validation of the results is required to ensure clinical relevance. Palmisano et al. reported that DNA methylation of *MGMT* was detectable in squamous cell lung carcinoma 3 years before clinical diagnosis [72]. Miglio et al. detected *MGMT* promoter methylation in 1 sample of sputum and 6 out of 8 bronchial washings from patients with small-cell lung cancer (SCLC) [73]. Agustí et al. reported that the induced sputum from peripheral lung cancer patients provided specimens of higher integrity and better diagnostic value [74].

3.5. Breast Cancer. Breast cancer is the most frequently diagnosed cancer in women, with the highest incidence (43.3 per 100,000) in women [1]. It affects individuals at a younger age compared to other majorly diagnosed cancers [1]. Conclusive detection of breast cancer is carried out using mammography, which has a sensitivity and specificity greater than 70% and 85% with larger tumours [8]. However, if the tumour is less than 1 cm, the sensitivity declined and epigenetic biomarkers could facilitate diagnosis in this subgroup [8].

Visvanathan et al. developed an epigenetic gene panel of 6 genes: *AKR1B1*, *HOXB4*, *RASGRF2*, *RASSF1*, *HIST1H3C*, and *TM6SF1* to predict survival in the early stages of metastatic breast cancer [75]. The serum from 141 women with metastatic breast cancer showed higher levels of methylation in patients with longer median progression free and overall survival [75]. Yamamoto et al. established a more efficient method to detect DNA methylation in serum using the one-step methylation-specific polymerase chain reaction (OS-MSP). They found promoter methylation of *GSTP1*, *RASSF1A*, and *RARb2* using 101 patients with primary breast cancer, 58 with metastatic breast cancer, and 87 healthy controls. They also determined higher sensitvity of these markers in early-stage primary tumour when compared with the conventional markers CEA and/or CA15-3. The combination of conventional markers with the panel of three epigenetic markers has an improved sensitivity to detect metastatic breast cancer [44]. Shan et al. developed a 6-gene panel using MethyLight to test for methylation in serum [8] and found that *SFN*, *hMLH1*, *HOXD13*, *PCDHGB7*, *RASSF1*, and *P16* were methylated in breast cancer patient serum [8]. Promoter methylation in this panel was correlated to patients with a family history of tumours and inversely correlated with proliferative index (ki-67). This study included serum from 268 patients with breast cancer, 236 patients with benign breast abnormalities, and 245 healthy volunteers [8]. Liu et al. reconfirmed *BRCA1* was not significantly methylated in breast cancer serum samples; however, hypermethylation of the *FHIT* was significantly higher in individuals with breast ductal carcinoma compared to healthy controls and those with benign breast tumours [76]. This study assessed gene methylation using the bisulfite sequencing method and high-resolution melting curve analysis in the serum of 36 patients with breast ductal carcinoma, 30 with benign breast fibroadenoma and 30 healthy volunteers [76]. Hagrass et al. studied a cohort of Egyptian women and discovered that promoter hypermethylation of the *estrogen receptor alpha* (*ERα*) was most frequently detected in individuals with breast cancer when tested in the serum of 120 patients with breast cancer compared to 100 benign breast lesions [77].

3.6. Ovarian Cancer. Ovarian cancer is a rare cancer affecting women and the most lethal gynecological malignancy [78, 79]. *RASSF1A* promoter methylation has been identified by numerous studies in the early stages of carcinogenesis in ovarian cancer tissue [80]. Giannopoulou et al. evaluated hypermethylation of *RASSF1A* in plasma and tissue samples collected from 53 patients with high-grade serous ovarian cancer, using real-time MSP with 62.3% of plasma samples showing a correspondence in *RASSF1A* methylation to matched ovarian cancer tissue [81]. Similarly, Wu et al. evaluated promoter methylation of *RASSF2A* and detected hypermethylation in 36.2% of plasma samples from ovarian cancer patients and hypermethylation in 51.1% in paired tissue samples, which was absent in plasma samples of 14 patients with benign disease and 10 normal controls [82]. Flanagan et al. identified that DNA methylation is promoted following platinum-based chemotherapy in the blood of 247 ovarian cancer patients and is associated with survival using methylation arrays and bisulfite pyrosequencing [83].

Current research has focused on epigenetic changes in malignant tissue, and there is a need for further investigation into noninvasive biomarkers for ovarian cancer.

4. Conclusion

Epigenetic biomarkers are a promising area of research with DNA methylation having the potential to provide a wealth of information regarding physiological and pathological status. Different stages and types of cancer produce a unique epigenetic signature. Methylation signatures can be implemented as specific and accurate biomarkers to establish tumour type and assist with prognosis and cancer management. Importantly, epigenetic markers can assist in the detection of cancers from the early stages, making them a favorable addition to the current set of detection methods used in the clinic. The studies reviewed here exemplify the recent research into DNA methylation including combinations of epigenetic markers which can produce an improved diagnostic power when compared to evaluating biomarkers individually. To facilitate the widespread use of epigenetic biomarkers in the clinic, the biomarkers in question and detection methods require standardisation for each cancer type. Currently, to the best of our knowledge, only *SEPT9* has received approval from the Food and Drug Administration (FDA) for use as a blood-based methylated biomarker for the diagnosis of colon cancer. Although other methylation-based biomarkers under investigation have been shown to have clinical relevance, further research is still necessary. These genes include *BRCA1* in breast cancer, *MGMT* in gliobastoma multiform (GBM), and *MLH1* in colon cancer. These biomarkers can enable the differentiation between tumour types in the clinic however still require invasive collection methods, and therefore, future developments of noninvasive methylation detection markers are needed. In conclusion, epigenetic alterations hold a great potential to become routine clinical cancer biomarkers due to their accuracy, specificity, and ease of collection, which justifies further research to implement standard panels of noninvasive epigenetic biomarkers to diagnose different malignancies.

Conflicts of Interest

All authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] Organization, W.H, *World Cancer Report 2014*, C. P. Wild and B. W. Stewart, Eds., 2014, http://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports/World-Cancer-Report-2014.

[2] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2016," *CA: a Cancer Journal for Clinicians*, vol. 66, no. 1, pp. 7–30, 2016.

[3] K. Warton and G. Samimi, "Methylation of cell-free circulating DNA in the diagnosis of cancer," *Frontiers in Molecular Biosciences*, vol. 2, p. 13, 2015.

[4] A. Bussieres, A. Bouchard, D. Simonyan, and S. Drolet, "Family medicine residents' training in, knowledge about, and perceptions of digital rectal examination," *Canadian Family Physician*, vol. 63, no. 4, pp. e232–e237, 2017.

[5] R. Cirincione, C. Lintas, D. Conte et al., "Methylation profile in tumor and sputum samples of lung cancer patients detected by spiral computed tomography: a nested case-control study," *International Journal of Cancer*, vol. 118, no. 5, pp. 1248–1253, 2006.

[6] M. Milosevic, Z. Jovanovic, and D. Jankovic, "A comparison of methods for three-class mammograms classification," *Technology and Health Care*, vol. 25, no. 4, pp. 657–670, 2017.

[7] P. B. Bach, J. N. Mirkin, T. K. Oliver et al., "Benefits and harms of CT screening for lung cancer: a systematic review," *JAMA*, vol. 307, no. 22, pp. 2418–2429, 2012.

[8] M. Shan, H. Yin, J. Li et al., "Detection of aberrant methylation of a six-gene panel in serum DNA for diagnosis of breast cancer," *Oncotarget*, vol. 7, no. 14, pp. 18485–18494, 2016.

[9] P. T. Hennessey, T. Sanford, A. Choudhary et al., "Serum microRNA biomarkers for detection of non-small cell lung cancer," *PLoS One*, vol. 7, no. 2, article e32307, 2012.

[10] T. A. Jatkoe, R. J. Karnes, S. J. Freedland, Y. Wang, A. Le, and J. Baden, "A urine-based methylation signature for risk stratification within low-risk prostate cancer," *British Journal of Cancer*, vol. 112, no. 5, pp. 802–808, 2015.

[11] J. D. Kuban, A. L. Tam, S. Y. Huang et al., "The effect of needle gauge on the risk of pneumothorax and chest tube placement after percutaneous computed tomographic (CT)-guided lung biopsy," *Cardiovascular and Interventional Radiology*, vol. 38, no. 6, pp. 1595–1602, 2015.

[12] N. M. Woody, K. L. Stephans, M. Andrews et al., "A histologic basis for the efficacy of SBRT to the lung," *Journal of Thoracic Oncology*, vol. 12, no. 3, pp. 510–519, 2017.

[13] S. Saxonov, P. Berg, and D. L. Brutlag, "A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 5, pp. 1412–1417, 2006.

[14] Y. Wang, Z. Yu, T. Wang, J. Zhang, L. Hong, and L. Chen, "Identification of epigenetic aberrant promoter methylation of RASSF1A in serum DNA and its clinicopathological significance in lung cancer," *Lung Cancer*, vol. 56, no. 2, pp. 289–294, 2007.

[15] Y. Y. Cheng, J. Yu, Y. P. Wong et al., "Frequent epigenetic inactivation of secreted frizzled-related protein 2 (SFRP2) by promoter methylation in human gastric cancer," *British Journal of Cancer*, vol. 97, no. 7, pp. 895–901, 2007.

[16] J. Yu, Q. Tao, Y. Y. Cheng et al., "Promoter methylation of the Wnt/beta-catenin signaling antagonist Dkk-3 is associated with poor survival in gastric cancer," *Cancer*, vol. 115, no. 1, pp. 49–60, 2009.

[17] G. O. Elliott, I. T. Johnson, J. Scarll et al., "Quantitative profiling of CpG island methylation in human stool for colorectal

cancer detection," *International Journal of Colorectal Disease*, vol. 28, no. 1, pp. 35–42, 2013.

[18] A. Linton, Y. Y. Cheng, K. Griggs et al., "An RNAi-based screen reveals PLK1, CDK1 and NDC80 as potential therapeutic targets in malignant pleural mesothelioma," *British Journal of Cancer*, vol. 110, no. 2, pp. 510–519, 2014.

[19] X. Yang, W. Dai, D. L. Kwong et al., "Epigenetic markers for noninvasive early detection of nasopharyngeal carcinoma by methylation-sensitive high resolution melting," *International Journal of Cancer*, vol. 136, no. 4, pp. E127–E135, 2015.

[20] A. Herbst, M. Wallner, K. Rahmig et al., "Methylation of helicase-like transcription factor in serum of patients with colorectal cancer is an independent predictor of disease recurrence," *European Journal of Gastroenterology & Hepatology*, vol. 21, no. 5, pp. 565–569, 2009.

[21] M. Brait, M. Banerjee, L. Maldonado et al., "Promoter methylation of MCAM, ERalpha and ERbeta in serum of early stage prostate cancer patients," *Oncotarget*, vol. 8, no. 9, pp. 15431–15440, 2017.

[22] Q. K. Deng, Y. G. Lei, Y. L. Lin, J. G. Ma, and W. P. Li, "Prognostic value of Protocadherin10 (PCDH10) methylation in serum of prostate cancer patients," *Medical Science Monitor*, vol. 22, pp. 516–521, 2016.

[23] A. Feber, P. Dhami, L. Dong et al., "UroMark-a urinary biomarker assay for the detection of bladder cancer," *Clinical Epigenetics*, vol. 9, p. 8, 2017.

[24] Y. Wang, Y. Yu, R. Ye et al., "An epigenetic biomarker combination of PCDH17 and POU4F2 detects bladder cancer accurately by methylation analyses of urine sediment DNA in Han Chinese," *Oncotarget*, vol. 7, no. 3, pp. 2754–2764, 2016.

[25] M. R. Abern, R. Owusu, and B. A. Inman, "Clinical performance and utility of a DNA methylation urine test for bladder cancer," *Urologic Oncology*, vol. 32, no. 1, pp. 51.e21–51.e26, 2014.

[26] M. O. Hoque, O. Topaloglu, S. Begum et al., "Quantitative methylation-specific polymerase chain reaction gene patterns in urine sediment distinguish prostate cancer patients from control subjects," *Journal of Clinical Oncology*, vol. 23, no. 27, pp. 6569–6575, 2005.

[27] O. E. Bryzgunova, E. S. Morozkin, S. V. Yarmoschuk, V. V. Vlassov, and P. P. Laktionov, "Methylation-specific sequencing of GSTP1 gene promoter in circulating/extracellular DNA from blood and urine of healthy donors and prostate cancer patients," *Annals of the New York Academy of Sciences*, vol. 1137, pp. 222–225, 2008.

[28] S. R. Payne, J. Serth, M. Schostak et al., "DNA methylation biomarkers of prostate cancer: confirmation of candidates and evidence urine is the most sensitive body fluid for non-invasive detection," *Prostate*, vol. 69, no. 12, pp. 1257–1269, 2009.

[29] Y. Su, H. Fang, and F. Jiang, "Integrating DNA methylation and microRNA biomarkers in sputum for lung cancer detection," *Clinical Epigenetics*, vol. 8, p. 109, 2016.

[30] A. J. Hubers, M. A. van der Drift, C. F. Prinsen et al., "Methylation analysis in spontaneous sputum for lung cancer diagnosis," *Lung Cancer*, vol. 84, no. 2, pp. 127–133, 2014.

[31] L. Guzman, M. S. Depix, A. M. Salinas et al., "Analysis of aberrant methylation on promoter sequences of tumor suppressor genes and total DNA in sputum samples: a promising tool for early detection of COPD and lung cancer in smokers," *Diagnostic Pathology*, vol. 7, p. 87, 2012.

[32] C. H. Mun, Y. Jung, S. H. Kim et al., "Three-dimensional electrospun poly(lactide-co-ε-caprolactone) for small-diameter vascular grafts," *Tissue Engineering Part A*, vol. 18, no. 15-16, pp. 1608–1616, 2012.

[33] J. Schussel, X. C. Zhou, Z. Zhang et al., "EDNRB and DCC salivary rinse hypermethylation has a similar performance as expert clinical examination in discrimination of oral cancer/dysplasia versus benign lesions," *Clinical Cancer Research*, vol. 19, no. 12, pp. 3268–3275, 2013.

[34] C. H. Chen, S. L. Yan, T. H. Yang et al., "The relationship between the methylated Septin-9 DNA blood test and stool occult blood test for diagnosing colorectal cancer in Taiwanese people," *Journal of Clinical Laboratory Analysis*, vol. 31, no. 1, 2017.

[35] W. H. Li, H. Zhang, Q. Guo et al., "Detection of SNCA and FBN1 methylation in the stool as a biomarker for colorectal cancer," *Disease Markers*, vol. 2015, Article ID 657570, 6 pages, 2015.

[36] T. Kadiyska and A. Nossikoff, "Stool DNA methylation assays in colorectal cancer screening," *World Journal of Gastroenterology*, vol. 21, no. 35, pp. 10057–10061, 2015.

[37] J. B. Kisiel, T. C. Yab, W. R. Taylor et al., "Stool DNA testing for the detection of pancreatic cancer: assessment of methylation marker candidates," *Cancer*, vol. 118, no. 10, pp. 2623–2631, 2012.

[38] Q. Tang, J. Cheng, X. Cao, H. Surowy, and B. Burwinkel, "Blood-based DNA methylation as biomarker for breast cancer: a systematic review," *Clinical Epigenetics*, vol. 8, p. 115, 2016.

[39] L. Van De Voorde, R. Speeckaert, D. Van Gestel et al., "DNA methylation-based biomarkers in serum of patients with breast cancer," *Mutation Research*, vol. 751, no. 2, pp. 304–325, 2012.

[40] T. deVos, R. Tetzner, F. Model et al., "Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer," *Clinical Chemistry*, vol. 55, no. 7, pp. 1337–1346, 2009.

[41] K. L. Mahon, W. Qu, J. Devaney et al., "Methylated glutathione S-transferase 1 (mGSTP1) is a potential plasma free DNA epigenetic marker of prognosis and response to chemotherapy in castrate-resistant prostate cancer," *British Journal of Cancer*, vol. 111, no. 9, pp. 1802–1809, 2014.

[42] T. H. Lee, L. Montalvo, V. Chrebtow, and M. P. Busch, "Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma," *Transfusion*, vol. 41, no. 2, pp. 276–282, 2001.

[43] N. Fujita, N. Kagara, N. Yamamoto et al., "Methylated DNA and high total DNA levels in the serum of patients with breast cancer following neoadjuvant chemotherapy are predictive of a poor prognosis," *Oncology Letters*, vol. 8, no. 1, pp. 397–403, 2014.

[44] N. Yamamoto, T. Nakayama, M. Kajita et al., "Detection of aberrant promoter methylation of GSTP1, RASSF1A, and RARβ2 in serum DNA of patients with breast cancer by a newly established one-step methylation-specific PCR assay," *Breast Cancer Research and Treatment*, vol. 132, no. 1, pp. 165–173, 2012.

[45] M. G. Friedrich, D. J. Weisenberger, J. C. Cheng et al., "Detection of methylated apoptosis-associated genes in urine sediments of bladder cancer patients," *Clinical Cancer Research*, vol. 10, no. 22, pp. 7457–7465, 2004.

[46] K. Yamamura, K. Kosumi, Y. Baba et al., "LINE-1 methylation level and prognosis in pancreas cancer: pyrosequencing technology and literature review," *Surgery Today*, 2017.

[47] J. Yu, T. Zhu, Z. Wang et al., "A novel set of DNA methylation markers in urine sediments for sensitive/specific detection of bladder cancer," *Clinical Cancer Research*, vol. 13, no. 24, pp. 7296–7304, 2007.

[48] S. Kurdyukov and M. Bullock, "DNA methylation analysis: choosing the right method," *Biology (Basel)*, vol. 5, no. 1, 2016.

[49] J. Tost and I. G. Gut, "DNA methylation analysis by pyrosequencing," *Nature Protocols*, vol. 2, no. 9, pp. 2265–2275, 2007.

[50] E. Olkhov-Mitsel and B. Bapat, "Strategies for discovery and validation of methylated and hydroxymethylated DNA biomarkers," *Cancer Medicine*, vol. 1, no. 2, pp. 237–260, 2012.

[51] S. J. Assinder and V. Bhoopalan, "A promising future for prostate cancer diagnostics," *Diagnostics (Basel)*, vol. 7, no. 1, 2017.

[52] L. Wang, Y. L. Lin, B. Li, Y. Z. Wang, W. P. Li, and J. G. Ma, "Aberrant promoter methylation of the cadherin 13 gene in serum and its relationship with clinicopathological features of prostate cancer," *The Journal of International Medical Research*, vol. 42, no. 5, pp. 1085–1092, 2014.

[53] K. Woodson, K. J. O'Reilly, J. C. Hanson, D. Nelson, E. L. Walk, and J. A. Tangrea, "The usefulness of the detection of GSTP1 methylation in urine as a biomarker in the diagnosis of prostate cancer," *The Journal of Urology*, vol. 179, no. 2, pp. 508–511, 2008, discussion 511-2.

[54] D. S. Millar, K. K. Ow, C. L. Paul, P. J. Russell, P. L. Molloy, and S. J. Clark, "Detailed methylation analysis of the glutathione S-transferase pi (GSTP1) gene in prostate cancer," *Oncogene*, vol. 18, no. 6, pp. 1313–1324, 1999.

[55] K. Daniunaite, A. Berezniakovas, F. Jankevičius, A. Laurinavičius, J. R. Lazutka, and S. Jarmalaitė, "Frequent methylation of RASSF1 and RARB in urine sediments from patients with early stage prostate cancer," *Medicina (Kaunas, Lithuania)*, vol. 47, no. 3, pp. 147–153, 2011.

[56] A. J. Edmondson, J. C. Birtwistle, J. W. F. Catto, and M. Twiddy, "The patients' experience of a bladder cancer diagnosis: a systematic review of the qualitative evidence," *Journal of Cancer Survivorship*, vol. 11, no. 4, pp. 453–461, 2017.

[57] O. Sanli, J. Dobruch, M. A. Knowles et al., "Bladder cancer," *Nature Reviews Disease Primers*, vol. 3, article 17022, 2017.

[58] M. O. Hoque, S. Begum, O. Topaloglu et al., "Quantitation of promoter methylation of multiple genes in urine DNA and bladder cancer detection," *Journal of the National Cancer Institute*, vol. 98, no. 14, pp. 996–1004, 2006.

[59] Y. Liu, M. H. Chew, C. K. Tham, C. L. Tang, S. Y. Ong, and Y. Zhao, "Methylation of serum SST gene is an independent prognostic marker in colorectal cancer," *American Journal of Cancer Research*, vol. 6, no. 9, pp. 2098–2108, 2016.

[60] D. K. Rex, C. Hassan, and M. J. Bourke, "The colonoscopist's guide to the vocabulary of colorectal neoplasia: histology, morphology, and management," *Gastrointestinal Endoscopy*, vol. 86, no. 2, pp. 253–263, 2017.

[61] A. Shirahata, K. Sakuraba, T. Goto et al., "Detection of vimentin (VIM) methylation in the serum of colorectal cancer patients," *Anticancer Research*, vol. 30, no. 12, pp. 5015–5018, 2010.

[62] K. Hibi, H. Mizukami, M. Saito, G. Kigawa, H. Nemoto, and Y. Sanada, "FBN2 methylation is detected in the serum of colorectal cancer patients with hepatic metastasis," *Anticancer Research*, vol. 32, no. 10, pp. 4371–4374, 2012.

[63] C. Tham, M. Chew, R. Soong et al., "Postoperative serum methylation levels of TAC1 and SEPT9 are independent predictors of recurrence and survival of patients with colorectal cancer," *Cancer*, vol. 120, no. 20, pp. 3131–3141, 2014.

[64] A. Semaan, A. van Ellen, S. Meller et al., "SEPT9 and SHOX2 DNA methylation status and its utility in the diagnosis of colonic adenomas and colorectal adenocarcinomas," *Clinical Epigenetics*, vol. 8, p. 100, 2016.

[65] A. Shirahata and K. Hibi, "Serum vimentin methylation as a potential marker for colorectal cancer," *Anticancer Research*, vol. 34, no. 8, pp. 4121–4125, 2014.

[66] N. M. Forones and M. Tanaka, "CEA and CA 19-9 as prognostic indexes in colorectal cancer," *Hepato-Gastroenterology*, vol. 46, no. 26, pp. 905–908, 1999.

[67] S. C. Glockner, M. Dhir, J. M. Yi et al., "Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer," *Cancer Research*, vol. 69, no. 11, pp. 4691–4699, 2009.

[68] J. W. Cui, W. Li, F. J. Han, and Y. D. Liu, "Screening for lung cancer using low-dose computed tomography: concerns about the application in low-risk individuals," *Translational Lung Cancer Research*, vol. 4, no. 3, pp. 275–286, 2015.

[69] J. A. Eggert, M. Palavanzadeh, and A. Blanton, "Screening and early detection of lung cancer," *Seminars in Oncology Nursing*, vol. 33, no. 2, pp. 129–140, 2017.

[70] S. M. Lee, J. Y. Park, and D. S. Kim, "Methylation of TMEFF2 gene in tissue and serum DNA from patients with non-small cell lung cancer," *Molecules and Cells*, vol. 34, no. 2, pp. 171–176, 2012.

[71] S. A. Belinsky, M. J. Grimes, E. Casas et al., "Predicting gene promoter methylation in non-small-cell lung cancer by evaluating sputum and serum," *British Journal of Cancer*, vol. 96, no. 8, pp. 1278–1283, 2007.

[72] W. A. Palmisano, K. K. Divine, G. Saccomanno et al., "Predicting lung cancer by detecting aberrant promoter methylation in sputum," *Cancer Research*, vol. 60, no. 21, pp. 5954–5958, 2000.

[73] U. Miglio, R. Mezzapelle, A. Paganotti et al., "Frequency of O^6-methylguanine-DNA methyltransferase promoter methylation in cytological samples from small cell lung cancer," *Diagnostic Cytopathology*, vol. 43, no. 11, pp. 947–952, 2015.

[74] C. Agustí, A. Xaubet, C. Montón et al., "Induced sputum in the diagnosis of peripheral lung cancer not visible endoscopically," *Respiratory Medicine*, vol. 95, no. 10, pp. 822–828, 2001.

[75] K. Visvanathan, M. S. Fackler, Z. Zhang et al., "Monitoring of serum DNA methylation as an early independent marker of response and survival in metastatic breast cancer: TBCRC 005 prospective biomarker study," *Journal of Clinical Oncology*, vol. 35, no. 7, pp. 751–758, 2017.

[76] L. Liu, L. Sun, C. Li et al., "Quantitative detection of methylation of FHIT and BRCA1 promoters in the serum of ductal breast cancer patients," *Bio-medical Materials and Engineering*, vol. 26, Supplement 1, pp. S2217–S2222, 2015.

[77] H. A. Hagrass, H. F. Pasha, and A. M. Ali, "Estrogen receptor alpha (ERα) promoter methylation status in tumor and serum DNA in Egyptian breast cancer patients," *Gene*, vol. 552, no. 1, pp. 81–86, 2014.

[78] A. Jemal, R. Siegel, J. Xu, and E. Ward, "Cancer statistics, 2010," *CA: a Cancer Journal for Clinicians*, vol. 60, no. 5, pp. 277–300, 2010.

[79] N. Colombo, M. Peiretti, G. Parma et al., "Newly diagnosed and relapsed epithelial ovarian carcinoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up," *Annals of Oncology*, vol. 21, Supplement 5, pp. v23–v30, 2010.

[80] J. G. Si, Y. Y. Su, Y. H. Han, and R. H. Chen, "Role of RASSF1A promoter methylation in the pathogenesis of ovarian cancer: a meta-analysis," *Genetic Testing and Molecular Biomarkers*, vol. 18, no. 6, pp. 394–402, 2014.

[81] I. C. Lydia Giannopoulou, K. Pavlakis, S. Kasimir-Bauer, and E. S. Lianidou, "RASSF1A promoter methylation in high-grade serous ovarian cancer: a direct comparison study in primary tumors, adjacent morphologically tumor cell-free tissues and paired circulating tumor DNA," *Oncotarget*, vol. 8, no. 13, pp. 21429–21443, 2017.

[82] Y. Wu, X. Zhang, L. Lin, X. P. Ma, Y. C. Ma, and P. S. Liu, "Aberrant methylation of RASSF2A in tumors and plasma of patients with epithelial ovarian cancer," *Asian Pacific Journal of Cancer Prevention*, vol. 15, no. 3, pp. 1171–1176, 2014.

[83] J. M. Flanagan, A. Wilson, C. Koo et al., "Platinum-based chemotherapy induces methylation changes in blood DNA associated with overall survival in patients with ovarian cancer," *Clinical Cancer Research*, vol. 23, no. 9, pp. 2213–2222, 2017.

[84] I. Renard, S. Joniau, B. van Cleynenbreugel et al., "Identification and validation of the methylated TWIST1 and NID2 genes through real-time methylation-specific polymerase chain reaction assays for the noninvasive detection of primary bladder cancer in urine samples," *European Urology*, vol. 58, no. 1, pp. 96–104, 2010.

[85] L. J. Bosch, S. Mongera, J. S. Terhaar Sive Droste et al., "Analytical sensitivity and stability of DNA methylation testing in stool samples for colorectal cancer detection," *Cellular Oncology (Dordrecht)*, vol. 35, no. 4, pp. 309–315, 2012.

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Research Article

Identification of Biomarkers for Predicting Lymph Node Metastasis of Stomach Cancer Using Clinical DNA Methylation Data

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Background. Lymph node (LN) metastasis was an independent risk factor for stomach cancer recurrence, and the presence of LN metastasis has great influence on the overall survival of stomach cancer patients. Thus, accurate prediction of the presence of lymph node metastasis can provide guarantee of credible prognosis evaluation of stomach cancer patients. Recently, increasing evidence demonstrated that the aberrant DNA methylation first appears before symptoms of the disease become clinically apparent. *Objective*. Selecting key biomarkers for LN metastasis presence prediction for stomach cancer using clinical DNA methylation based on a machine learning method. *Methods*. To reduce the overfitting risk of prediction task, we applied a three-step feature selection method according to the property of DNA methylation data. *Results*. The feature selection procedure extracted several cancer-related and lymph node metastasis-related genes, such as TP73, PDX1, FUT8, HOXD1, NMT1, and SEMA3E. The prediction performance was evaluated on the public DNA methylation dataset. The results showed that the three-step feature procedure can largely improve the prediction performance and implied the reliability of the biomarkers selected. *Conclusions*. With the selected biomarkers, the prediction method can achieve higher accuracy in detecting LN metastasis and the results also proved the reliability of the selected biomarkers indirectly.

1. Introduction

According to the recent reports of the World Health Organization (WHO), stomach cancer is the fifth most common cancer in the world and more than 70% of the new cases of stomach cancer occurred in developing countries (mainly in China) [1, 2]. The early stage of stomach cancer, which is defined as stomach cancer limited to the mucosa or submucosa and irrelevant to the presence or absence of lymph node (LN) metastasis, confers a survival rate of greater than 90% in 5 years in many centers [3]. However, even in the early stage, it was reported that the incidence of LN metastasis was 14.1% overall and was 4.8 to 23.6% depending on cancer depth [4, 5]. Many researchers demonstrated that LN metastasis is an independent risk factor for stomach cancer recurrence in patients following curative resection, and the overall survival of LN metastasis-negative stomach cancer patient is significantly longer than that of LN metastasis-positive patients [6, 7]. Therefore, it is certain that an accurate LN metastasis presence prediction can provide the guarantee of credible prognosis evaluation of stomach cancer patients.

Traditionally, LN metastasis diagnosis is mainly implemented by preoperative imaging such as abdominal ultrasonography (US) and computed tomography (CT), but their diagnostic accuracy is limited. It was reported that the detection rate of lymph nodes around the stomach was 18.7% in CT and 5.0% in US [8]. Endoscopic

ultrasonography (EUS) is an effective approach and generally provides a more accurate prediction of the tumor stage than does CT. However, EUS-based prediction accuracy for LN is only slightly greater as compared to CT [4].

Recently, increasing evidences suggest the critical role of DNA methylation in human carcinogenesis [9, 10]. Aberrant DNA methylation is one of the common alterations in carcinogenesis, and it first appears before symptoms of the disease become clinically apparent [11–13]. In addition, aberrant DNA methylation can promote the progression of disease [14]. With the development of high-throughput technology, plenty of DNA methylation data are available for cancer prediction and biomarker identification [15–18]. Inspired by these applications, in this study, we used the DNA methylation data to categorize the incidence of LN metastasis in stomach cancer through a machine learning method. Considering the high-dimensionality and high-noisiness of the DNA methylation data, there are still several challenges to achieve the categorization. In contrast to the large number of features (probes), the small number of cancer samples available for training may lead to the degradation of classification performance and raise the risk of overfitting [19]. It is natural and perhaps essential to employ a feature selection step to obtain a feature set which only consists of genes contributing positively to the classification without redundant features. The key benefits of performing feature selection are reducing overfitting, improving accuracy, and reducing training time. Beyond that, feature selection in cancer research can help researchers to identify key carcinogenic markers and accurate prediction can provide references for clinical implementation. The feature selection methods mainly can be divided into three categories, which are the filter, wrapper, and embedded methods [20–23]. The filter methods use a measure to score feature subsets while the wrapper methods use a predictive model to score. With the wrapper method, different feature sets are generated and an optimal engine, such as genetic method [24], simulated annealing method [25], and particle swarm optimization method [26], is selected to search a set of features that best distinguish the training samples of different classes. Embedded methods are the catch-all group of techniques which perform feature selection as part of the model learning process.

In this study, we grouped the data of stomach cancer into three categories, normal, LN metastasis negative, and LN metastasis positive, according to the clinical information. A three-step feature selection method was applied to identify the key genes. To evaluate the reliability of the selected biomarkers, we introduced the random forest algorithm to predict the categories with and without the three-step feature selection method. The results showed that the prediction accuracy was largely improved with the selected biomarkers, and it also proved the reliability indirectly.

2. Results

2.1. Feature Selection. Feature selection is commonly used to remove the irrelevant and redundant features from the original feature set. The minimum redundancy maximum relevance (mRMR) feature selection method is a feature selection method for finding a set of features that have the highest relevance with the target class and are also maximally dissimilar to each other based on the mutual information theory. However, mRMR is computationally expensive. In our paper, the differential methylation analysis was integrated with mRMR to achieve the preliminary feature selection. To further obtain the most informative feature for classification, an embedded feature selection method with genetic algorithm was introduced to get the final optimal features.

2.1.1. Feature Selection with Differential Methylation Region (DMR) Analysis. To preliminarily obtain the probes that are closely related to the phenotype, DMR analysis, which aimed to identify significantly methylated probes between different phenotypes, was applied. We compared the methylation status of each probes in the normal samples within the cancer samples and the methylation status of probes in the LN-negative samples within the LN-positive samples. Differentially methylated probes were determined with the Mann–Whitney U test. The density of the mean difference and the Benjamin-Hochberg- (BH-) adjusted p value of the two comparisons were shown in Figure 1, from which we can see that the methylation patterns were much more similar in the LN-negative and LN-positive samples than in the normal samples and cancer samples. The appearance indicated that the thresholds used for selecting significantly differentially methylated probes must be different according to the two comparisons. For the comparison of normal versus cancer, we selected probes with an adjusted p value less than $1E{-}5$ and an absolute mean difference greater than 0.2 as significantly differentially methylated probes. For the comparison of LN negative versus LN positive, the threshold for the adjusted p value and absolute mean difference was set as 0.01 and 0.02, respectively. With such criteria, we identified 1077 and 275 as significantly differentially methylated probes in the two comparisons. There were only 33 probes shared by both.

2.1.2. Feature Selection with the mRMR Method. The classic mRMR method was applied to filter the probes selected previously, and the probes were ranked according to their score. Since there is no explicit threshold, only the top 10% probes were left and these probes were used as input to the next feature selection step. The results of mRMR filtering were shown in Figure 2, from which we can see that the scores in respect to the LN negative versus LN positive comparison were extremely low. The results implied that the LN-negative samples and LN-positive samples were very indistinct.

2.1.3. Feature Selection with Genetic Algorithm. Performing feature selection with genetic algorithm requires conceptualizing the processing of feature selection as an optimization problem and encoded the solution as binary. In this paper, random forest algorithm was used as the fit function during the genetic algorithm and the receiver operating characteristic (ROC) was used to measure the fitness.

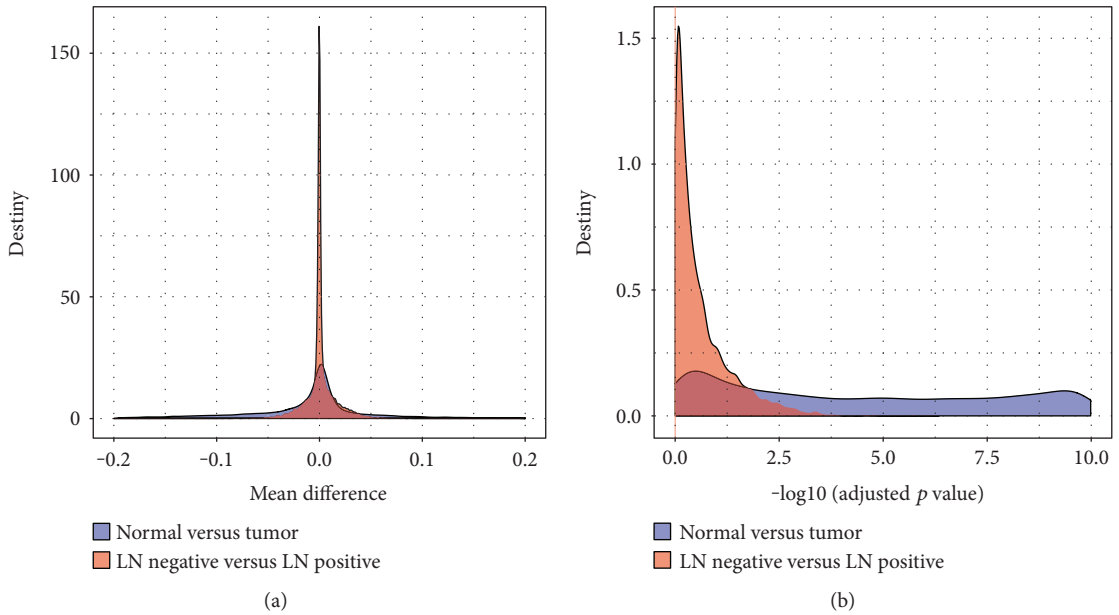

Figure 1: The density of the mean difference and BH-adjusted p value of the two comparisons. (a) The density of the mean difference of normal versus cancer comparison and LN negative versus LN positive comparison. (b) The density of the log10 BH-adjusted p value of normal versus cancer comparison and LN negative versus LN positive comparison.

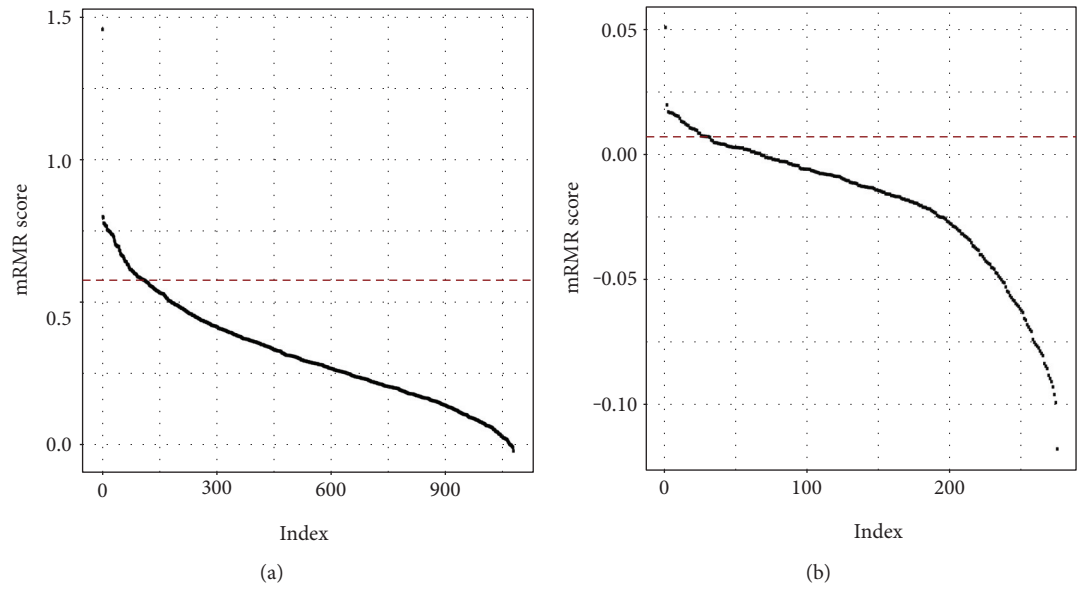

Figure 2: The distribution of mRMR scores with respect to features. The dashed line corresponds to the 10% cutoff used. (a) Normal versus cancer. (b) LN negative versus LN positive.

The details will be discussed later in the section of Materials and Methods. The normal versus cancer classification and LN negative versus LN positive classification were treated independently.

During the genetic algorithm in respect to the normal versus tumor classification, the ROC value summary in each iteration was shown in Figure 3(a), from which we can see that almost all the solutions can give a high fitness value. From this plot, we can see that after 12 iterations, the mean fitness hovered around 0.9999. We collected all the best solutions after each of the 12 iterations and simply summarized how many times a probe had been selected. The distribution of the number of selected probes were shown in Figure 3(b), and we selected the top 20 probes as the final features used for classification. According to the genomic locations, the 20 probes were associated to 39 genes including well-known cancer-related genes, such as *TP73*, *PDX1*, and *FUT8* [27–29].

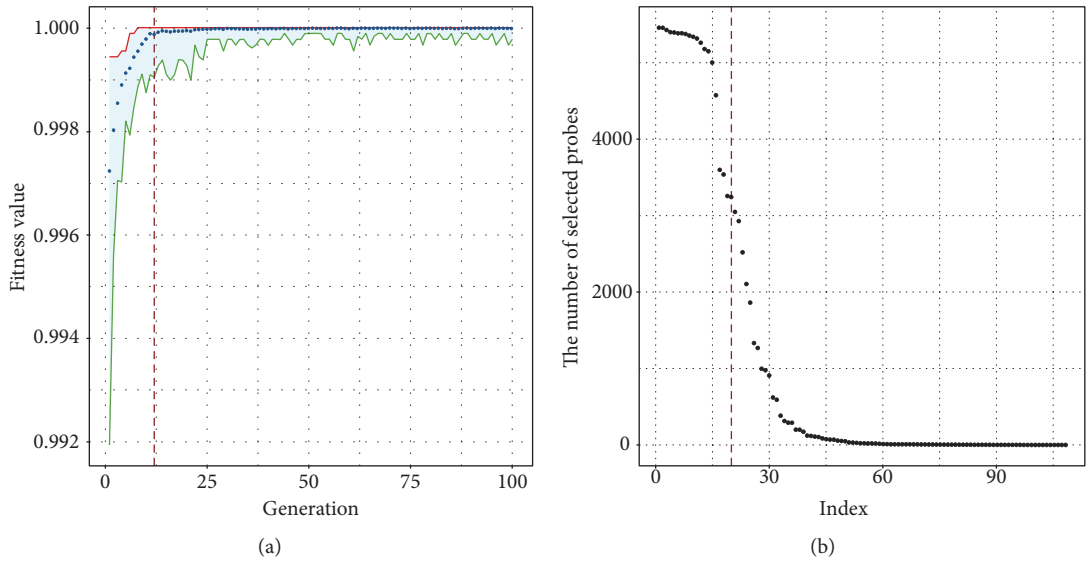

Figure 3: The results of genetic algorithm-based feature selection with respect to the normal versus tumor classification. (a) The fitness improvement in the process of iteration. (b) The distribution of the number of selected probes.

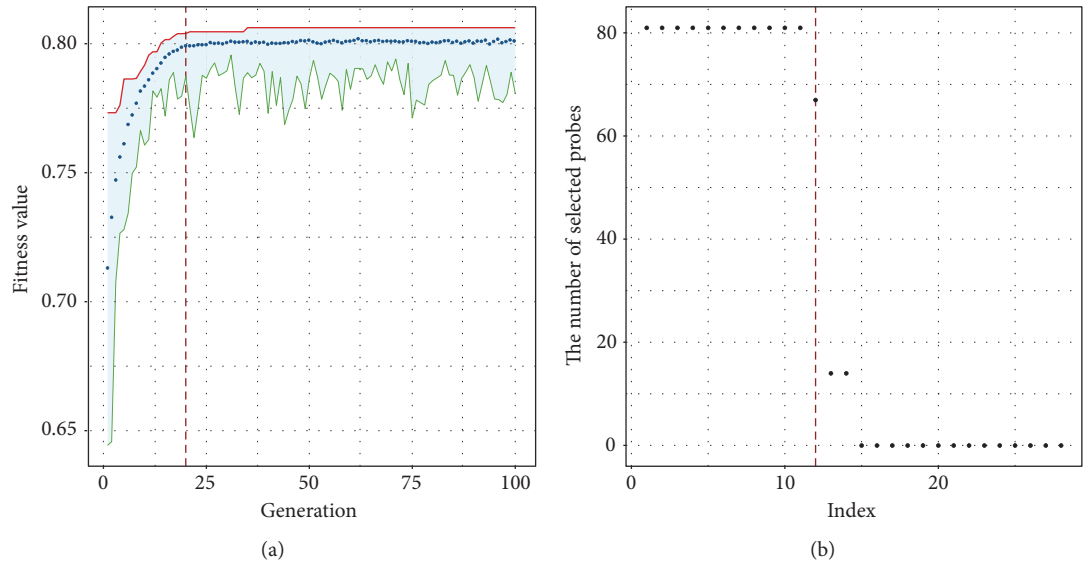

Figure 4: The results of genetic algorithm-based feature selection with respect to the LN negative versus LN positive classification. (b) The fitness improvement in the process of iteration. (a) The distribution of the number of selected probes.

The results of genetic algorithm in respect to the LN negative versus LN positive classification were shown in Figure 4(a), from which we can find that even after 100 iterations, the fitness is still not much greater than 0.8. This result also implied the indistinctness between the LN-negative and LN-positive samples. The mean fitness hovered around 0.8 after iteration 20. Similarly, we collected all the best solutions after each 20 iterations, and the distribution of the number of selected probes was shown in Figure 4(b). Finally, 12 probes were chosen for the final classification and associated with 14 genes including several lymph node metastasis-related genes, such as *HOXD1*, *NMT1*, and *SEMA3E* [30].

2.2. Classification Performance Evaluation. To illustrate the necessity and effectiveness of the feature selection procedure, we compared the performance of the random forest using the three-step-selected probes with the random forest using only the differentially methylated probes. We randomly generated 100 training and testing data for evaluation, and the AUROC (area under ROC curve) value was used as measurement. The AUROC value of a classifier described the probability that the classifier will rank a randomly chosen positive instance higher than a randomly chosen negative instance. Simply put that a larger value of the AUROC means a higher discriminatory power. The box plots in Figure 5 shown below were the distribution of the AUC values of the prediction in

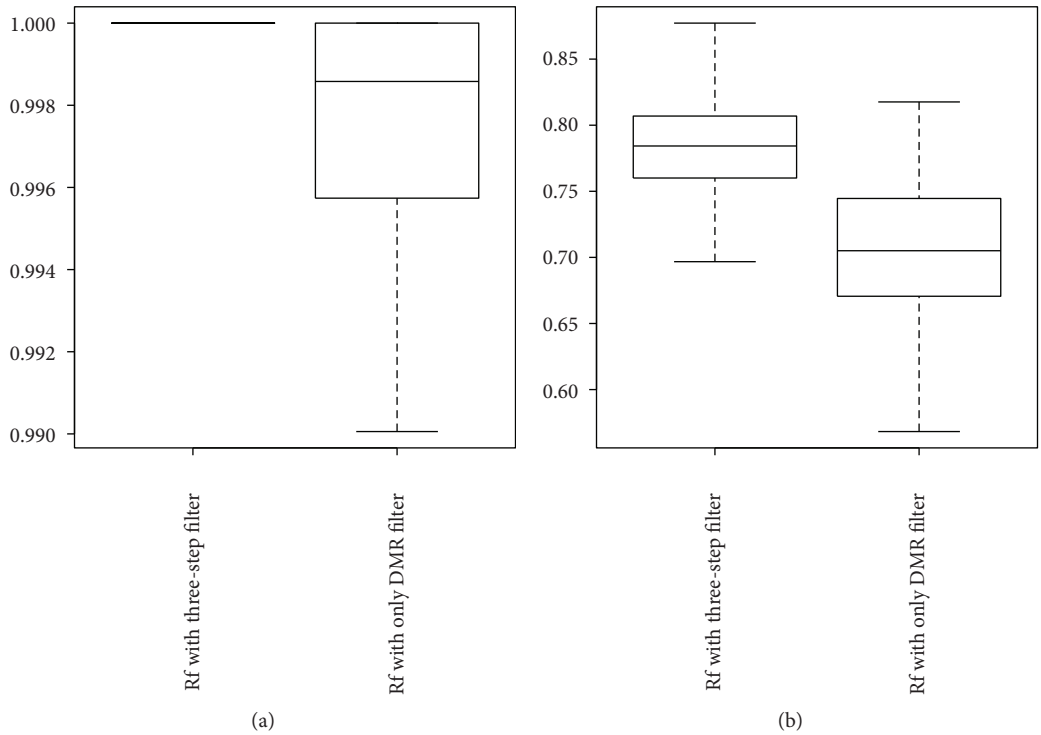

Figure 5: The distribution of the AUC value with different methods. (a) AUC value with different methods with respect to the normal versus tumor classification. (b) AUC value with different methods with respect to the LN negative versus LN positive classification.

Table 1: The sample number for each phenotype.

Normal	Cancer		
	LN negative	LN positive	Unclassified
27	94	189	12

respect to the normal versus tumor and LN negative versus LN positive.

From the plots, we can see that with the three-step feature selection procedure, the classifier can give a better performance in respect to both the normal versus tumor and LN negative versus LN positive classifications compared to with only the DMR analysis. Moreover, we also can find that the three-step feature selection or DMR only analysis gives good performance (AUC value all greater than 0.99) for the normal versus tumor classification.

3. Materials and Methods

3.1. DNA Methylation Dataset and DMR Analysis. The clinical data and the TCGA level 3 DNA methylation data were downloaded from The Cancer Genome Atlas (TCGA) project [31]. Only the samples with clear clinical diagnosis were used in the study. The details were shown in Table 1.

To identify differentially methylated probes, for each probe, we ranked the samples and compared only the lower methylation quintile sample to the upper methylation quintile sample between two phenotypes using the Mann–Whitney U test. The BH-adjusted p value and mean methylation difference were used to guide the identification.

3.2. Genetic Algorithm. Genetic algorithms are optimization tools that search the solution through simulating the evolution of random variation and natural selection. For feature selection, the individuals are subsets of candidate features that are encoded as binary and the value indicated that a feature is either included or not in the subset. The parameters used for the genetic algorithm were set as follow [19]:

(i) Population size: 100

(ii) Maximum number of generations: 100

(iii) Selection method: tournament selection with size = 2

(iv) Elitism rate: 10 individuals

(v) Crossover: 2-point crossover with probability 0.6

(vi) Mutation: random mutation with probability 0.05

The initial population was created by producing chromosomes with a random 30% of the predictors. The fitness function of every individual was defined as the ROC value of the classification method.

4. Conclusions

Stomach cancer is the fifth most common cancer in the world, and most of the new cases occurred in developing countries, especially in China. Recently, more and more

Table 2: Identified biomarkers for each prediction.

Normal versus tumor biomarkers	LN negative versus LN positive biomarkers
SLC39A5, *C3orf32*, *TP73*, *CD1B*, *PCDHGA4*, *PCDHGA11*, *PCDHGA9*, *PCDHGA1*, *PCDHGB1*, *PCDHGB6*, *PCDHGA12*, *PCDHGB3*, *PCDHGB7*, *PCDHGA6*, *PCDHGA8*, *PCDHGA10*, *PCDHGA5*, *PCDHGB4*, *PCDHGA3*, *PCDHGA2*, *PCDHGB2*, *PCDHGA7*, *PCDHGB5*, *C20orf197*, *SLC16A5*, *FUT8*, *SLC15A2*, *C17orf93*, *PRAC*, *OCLN*, *TMEM144*, *FGF2*, *PDX1*, *CCL1*, *LILRB5*, *LCE3D*, *GPR45*, *LPO*, *CGB5*	*LAT2*, *TTC13*, *ARV1*, *NMT1*, *DCAKD*, *GJA1*, *OR7A17*, *LOX*, *KRT19*, *ZNF655*, *KRTAP4-4*, *TAAR5*, *SEMA3E*, *HOXD1*

evidence demonstrated that LN metastasis was an independent risk factor for stomach cancer recurrence in patients following curative resection, and the overall survival of LN metastasis-negative stomach cancer patients is significantly longer than that of LN metastasis-positive patients.

Based on the critical role of DNA methylation in human carcinogenesis, in this study, we focused on the prediction of the LN metastasis status using the DNA methylation data. However, considering the inherent disadvantage of DNA methylation data, such as the limited sample number compared to the large number of probes, we applied a three-step feature selection procedure to extract a small subset of representative features. First, we applied the differential methylation analysis to identify the significantly methylated probes between different phenotypes. Then, an mRMR method was introduced to remove the redundant feature obtained in the first filter step. Finally, a wrapper method based on genetic algorithm was used to achieve the final feature selection. We obtained 20 probes related to 39 genes which were inputs of the prediction in respect to normal versus tumor, and 12 probes related to 14 genes were input to the prediction in respect to LN negative versus LN positive (see Table 2). These genes related to the selected probes are mostly associated with cancer and LN metastasis, such as *TP73*, *PDX1*, *FUT8*, *HOXD1*, *NMT1*, and *SEMA3E*.

To evaluate the effect of three-step feature selection to the prediction performance, we downloaded the DNA methylation data and clinical data from the TCGA project. The AUROC value was used as the performance measurement. The experiment results showed that the three-step feature selection can largely improve the performance of prediction, especially predicting LN negative versus LN positive. The source code used in this paper can be obtained at https://git.oschina.net/junwu302/codes/m2gonkax18sfhdvl3e0b932.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit et al., "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer*, vol. 136, no. 5, pp. E359–E386, 2015.

[2] S. Antoni, I. Soerjomataram, B. Møller, F. Bray, and J. Ferlay, "An assessment of GLOBOCAN methods for deriving national estimates of cancer incidence," *Methods*, vol. 3, p. 4, 2016.

[3] E. Rubin and J. P. Palazzo, "The gastrointestinal tract," in *Rubin's Pathology. Clinicopathologic Foundations of Medicine*, pp. 660–739, Lippincott Williams & Wilkins, Philadelphia, 2005.

[4] T. Akagi, N. Shiraishi, and S. Kitano, "Lymph node metastasis of gastric cancer," *Cancer*, vol. 3, no. 2, pp. 2141–2159, 2011.

[5] Y. D. Park, Y. J. Chung, H. Y. Chung et al., "Factors related to lymph node metastasis and the feasibility of endoscopic mucosal resection for treating poorly differentiated adenocarcinoma of the stomach," *Endoscopy*, vol. 40, no. 1, pp. 7–10, 2008.

[6] R. M. Kwee and T. C. Kwee, "Predicting lymph node status in early gastric cancer," *Gastric Cancer*, vol. 11, no. 3, pp. 134–148, 2008.

[7] J. Y. Deng and H. Liang, "Clinical significance of lymph node metastasis in gastric cancer," *World Journal of Gastroenterology*, vol. 20, no. 14, pp. 3967–3975, 2014.

[8] H. Isozaki, K. Okajima, E. Nomura et al., "Preoperative diagnosis and surgical treatment for LN metastasis in gastric cancer (in Japanese)," *Gan to Kagaku Ryoho*, vol. 23, pp. 1275–1283, 1996.

[9] S. B. Baylin and J. G. Herman, "DNA alterations in cancer: genetic and epigenetic alterations," in *DNA Alterations in Cancer*, pp. 293–309, Eaton Publishing, Natick, 2000.

[10] M. Ehrlich, "DNA methylation in cancer: too much, but also too little," *Oncogene*, vol. 21, no. 35, pp. 5400–5413, 2002.

[11] Y. Bergman and H. Cedar, "DNA methylation dynamics in health and disease," *Nature Structural & Molecular Biology*, vol. 20, no. 3, pp. 274–281, 2013.

[12] S. B. Baylin, S. A. Belinsky, and J. G. Herman, "Aberrant methylation of gene promoters in cancer—concepts, misconcepts, and promise," *Journal of the National Cancer Institute*, vol. 92, no. 18, pp. 1460-1461, 2000.

[13] K. M. Godfrey, A. Sheppard, P. D. Gluckman et al., "Epigenetic gene promoter methylation at birth is associated with child's later adiposity," *Diabetes*, vol. 60, no. 5, pp. 1528–1534, 2011.

[14] A. Jones, A. E. Teschendorff, Q. Li et al., "Role of DNA methylation and epigenetic silencing of HAND2 in endometrial cancer development," *PLoS Medicine*, vol. 10, no. 11, article e1001551, 2013.

[15] W. A. Palmisano, K. K. Divine, G. Saccomanno et al., "Predicting lung cancer by detecting aberrant promoter methylation in sputum," *Cancer Research*, vol. 60, no. 21, pp. 5954–5958, 2000.

[16] P. Adorján, J. Distler, E. Lipscher et al., "Tumour class prediction and discovery by microarray-based DNA methylation analysis," *Nucleic Acids Research*, vol. 30, no. 5, article e21, 2002.

[17] F. J. Carmona, D. Azuara, A. Berenguer-Llergo et al., "DNA methylation biomarkers for noninvasive diagnosis of colorectal cancer," *Cancer Prevention Research*, vol. 6, no. 7, pp. 656–665, 2013.

[18] S. Fukushige and A. Horii, "DNA methylation in cancer: a gene silencing mechanism and the clinical potential of its biomarkers," *The Tohoku Journal of Experimental Medicine*, vol. 229, no. 3, pp. 173–185, 2013.

[19] H. Hijazi and C. Chan, "A classification framework applied to cancer gene expression profiles," *Journal of Healthcare Engineering*, vol. 4, no. 2, pp. 255–283, 2013.

[20] J. Bi, K. Bennett, and M. Embrechts, "Dimensionality reduction via sparse support vector machines," *Journal of Machine Learning Research*, vol. 3, pp. 1229–1243, 2003.

[21] R. Kohavi and G. H. John, "Wrappers for feature subset selection," *Artificial Intelligence*, vol. 97, no. 1, pp. 273–324, 1997.

[22] S. Perkins, K. Lacker, and J. Theiler, "Grafting: fast, incremental feature selection by gradient descent in function space," *Journal of Machine Learning Research*, vol. 3, pp. 1333–1356, 2003.

[23] I. Guyon and A. Elisseeff, "An introduction to variable and feature selection," *Journal of Machine Learning Research*, vol. 3, pp. 1157–1182, 2003.

[24] X. M. Zhao, Y. M. Cheung, and D. S. Huang, "A novel approach to extracting features from motif content and protein composition for protein sequence classification," *Neural Networks*, vol. 18, no. 8, pp. 1019–1028, 2005.

[25] H. Q. Wang, D. S. Huang, and B. Wang, "Optimisation of radial basis function classifiers using simulated annealing algorithm for cancer classification," *Electronics Letters*, vol. 41, no. 11, pp. 630–632, 2005.

[26] K. H. Chen, K. J. Wang, M. L. Tsai et al., "Gene selection for cancer identification: a decision tree model empowered by particle swarm optimization algorithm," *BMC Bioinformatics*, vol. 15, no. 1, p. 1, 2014.

[27] T. Stiewe and B. M. Putzer, "Role of p73 in malignancy: tumor suppressor or oncogene?," *Cell Death and Differentiation*, vol. 9, no. 3, pp. 237–245, 2002.

[28] J. Ma, M. Chen, J. Wang et al., "Pancreatic duodenal homeobox-1 (PDX1) functions as a tumor suppressor in gastric cancer," *Carcinogenesis*, vol. 29, no. 7, pp. 1327–1333, 2008.

[29] Y. Ito, A. Miyauchi, H. Yoshida et al., "Expression of α1, 6-fucosyltransferase (FUT8) in papillary carcinoma of the thyroid: its linkage to biological aggressiveness and anaplastic transformation," *Cancer Letters*, vol. 200, no. 2, pp. 167–172, 2003.

[30] S. Bhatlekar, J. Z. Fields, and B. M. Boman, "HOX genes and their role in the development of human cancers," *Journal of Molecular Medicine*, vol. 92, no. 8, pp. 811–823, 2014.

[31] "The TCGA Database," http://cancergenome.nih.gov/.

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Research Article

Noncoding Centromeric RNA Expression Impairs Chromosome Stability in Human and Murine Stem Cells

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We analyzed the effect of transcribed noncoding RNA centromeric satellites on chromosome segregation in normal human and murine stem and fibrosarcoma cells. The overexpression of different centromeric alphoid DNAs in all cell lines induced a marked increase in chromosome mis-segregation in anaphase. Overexpression of centromeric mouse minor satellite also increased chromosome instability in the murine stem but not in human cells. Analysis of chromosome segregation in vivo showed disturbances in the mitotic progression, which was frequently unresolved. Live cell imaging revealed that overexpression of centromeric satellites resulted in several different chromosomal morphological errors in the cell nuclei. Our findings correlated with other reports that several centromeric noncoding RNAs are detected in different carcinoma cells and their expression resulted in segregation errors. Our study furnishes further insights into a novel source of genomic instability in human and murine cells. It has recently been shown that noncoding centromeric RNAs are present in some form of cancer, and thus, overexpression of several types of centromeric noncoding RNAs may be useful as a specific maker for neoplastic cells.

1. Introduction

Repetitive satellite DNA sequences (alphoid DNA) are essential for centromere formation and function during cell division [1, 2]. The centromere protein (CENP) requirements that affect chromosome function and segregation are complex [3]. Factors such as noncoding RNAs (ncRNAs) formed from transcripts of centromeric satellite DNA also influence chromosome and chromatin organisation in human [4] and murine [5, 6] cells. Centromeric RNAs are closely associated with centromeric chromatin and kinetochore formation. Furthermore, human centromeric RNAs were found to be transcribed in several tumour types but not in normal somatic tissues, suggesting that ncRNAs may play a role in cancer establishment or progression [7, 8]. In recent years, growing evidence has shown that transcription of noncoding RNA from pericentric and centromeric satellites could lead to mitotic or segregation errors [9]. The dosage balance of the ncRNAs is important for correct cell cycle progression, and balance perturbation might result in malignancy [10].

Human chromosome centromeres are comprised of tandemly repeated arrays of alpha (alphoid, α) satellite DNA arranged as 171 bp monomer units. The monomer arrays are chromosome specific but share a high homology in consensus sequence between chromosomes [11, 12]. The mouse centromere-specific minor satellite DNA is different in that the arrays are highly homogeneous between chromosomes. The α satellite DNA (with the exception of chromosome Yα) and the murine minor satellite contain a 17 bp motif known as the CENP-B box that binds to centromeric protein B (CENP-B) [13, 14]. In this study, we investigated the role of ncRNAs transcribed from different chromosome-specific centromeric satellites on chromosome segregation. In tumour and immortalised cells, chromosome segregation is impaired compared to that in normal cells. The overexpression of centromeric alphoid satellite DNA from chromosomes

17, 21, and Y in human stem (HUES-10) and fibrosarcoma (HT1080) cells resulted in a marked increase in chromosome mis-segregation events during anaphase. The analysis of HT1080 cells overexpressing 17 alphoid DNA during live cell imaging showed the disturbances in mitotic progression from metaphase to anaphase, which usually resulted in cell death. In comparison, the overexpression of the noncentromeric human DYZ1 satellite and the control vector DNA had no effect on chromosome segregation in HUES-10 and HT1080 cells. The minor satellite ncRNAs impaired chromosome segregation only in murine stem (ES) cells, indicating that the effect is species specific.

This study is the first example of using a live cell imaging system to observe the morphological deformities in the cell nuclei, resulting from centromeric ncRNA overexpression. The results highlight the importance of centromeric ncRNA expression on chromosomal instability.

2. Materials and Methods

2.1. Satellite Expression Constructs. Six eukaryotic expression vectors containing different repetitive DNA sequences were constructed, with the vector pIRESneo2 (pIneo2, Clontech), as the backbone. Add a sentence about generating the vector. A 2.7 kb fragment of core human chromosome 17 alpha satellite was obtained from EcoRI digestion of hBAC227J24 BAC (Kim et al., 1996) and cloned into the EcoRI site of pIneo2, to obtain pI-17α. Similarly, pI21α was assembled by cloning into pIneo2, a 1.4 kb fragment of human chromosome 21 alpha satellite, released by EcoRI digestion of pHSV21αHPRT-Neo (Moralli et al., 2006). The human Y chromosome alpha satellite was obtained by PCR amplification of total human genomic DNA using a specific primer (5′-ATG ATA GAA ACG GAA ATA TG-3′ and 5′-AGT AGA ATG CAA AGG GCT CC-3′. The 800 bp PCR product was cloned into an intermediate vector using the T/A cloning system (Promega, pGEM-Teasy system), excised by EcoRI digestion as an 850 bp fragment, and ligated into pIneo2 to obtain pIYα. The noncentromeric repetitive satellite DYZ1 from the human Y chromosome was amplified by PCR on genomic DNA using the primer sequences DYZ1 1F 5′-TCC CAT CCA ATC CAA TCT AC-3′ and DYZ1 1R 5′-GGA GTG GAA TAG ACA AGA GT-3′. As described for pIYα, the 1.4 kb DYZ1 PCR fragment was cloned into an intermediate vector, excised by EcoRI digestion as a 1.3 kb fragment, and ligated into pIRESneo2 to obtain the pIDYZ1 vector. A 1.7 kb fragment of mouse centromeric minor satellites was amplified from genomic mouse DNA with primers 5′-AAA AAA AAG GAT CCA AAA TTT AGA AAT GTC CAC TG-3′ and 5′-AAA AAA AAA GCT TAA GAT CTC CAT ATT TCA CGT CC-3′ and cloned into pBeloBAC 11 into BamH1 and BglII sites. The insert was then removed from the resulting vector (MNR) by NotI digestion and ligated into the pIneo2 vector into the NotI site, to generate pI-Minor. The pI-Major vector was similarly produced by PCR cloning of a 3.2 kb fragment with major satellite-specific primers 5′-AAA AAA AAG GAT CCG TGA GTT ACA CTG AAA AAC-3′ and 5′-AAA AAA AAA GCT TAA GAT CTT CCC GTT TCC AAC G-3′ on pBeloBAC 11. The fragment was then released by the E3.2 intermediate construct by NotI digestion and ligated into pIneo2. The specificity of all satellite sequences was tested by FISH on either human or mouse cells, to ascertain that they labelled specific chromosomes. The pH2BmCherry was produced by excising GFP from pH2BGFP using AgeI/NotI digestion; mCherry was cut from pmCherry (Clontech) by the same digestion. The mCherry fragment was ligated into the pH2B vector backbone to obtain pH2BmCherry.

2.2. Cell Culture and Transfection. Human fibrosarcoma HT1080 cells were grown in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Mouse ES 14TG2A cells were grown in DMEM-GlutaMax with 15% fetal calf serum (FCS), 200 mM glutamine, 1× nonessential amino acids, 1% penicillin/streptomycin, 0.1 mM β-mercaptoethanol, and 1× leukemia inhibitory factor (Lif). Feeder-independent HUES-10 cells were grown on Matrigel (BD)-coated wells using the mTeSR medium (Stem Cell Technologies). TrypLE Express (Invitrogen) was used to enzymatically passage the hES cells. To increase single-cell survival, a ROCK inhibitor (Merck Biosciences) was added during each passaging step, at a final concentration of 10 μM. All cells were incubated in a 37°C incubator supplied with 5% CO_2. The day before transfection, 2×10^6 cells were seeded in 6-well dishes. For each vector, 3 μg of DNA was transfected into the target cells using 10 μl of ExGen 500 transfecting agent per dish (Fermentas); the plate was centrifuged at 280*g* for 5 minutes. The cells were incubated at 37°C in an incubator supplied with 5% CO_2.

2.3. Cell Fixation and Analysis of Segregation. After 72 hours, the cells were fixed for 10 minutes with 4% paraformaldehyde in PBS and counter-stained with DAPI. For each slide, 100 metaphases and anaphases were analyzed and the number and type of mis-segregation events scored. Each set of experiment was repeated at least three times. Fluorescence in situ hybridization (FISH) was carried out as described in (Moralli and Monaco, 2009). The slides were examined with an Olympus BX-51 epifluorescence microscope coupled to a JAI CVM4+ CCD camera. Images were acquired using Genus Software from CytoVision.

2.4. Immunostaining and FISH. Following transfection as outlined above, the cells were grown on glass slides and fixed in 4% paraformaldehyde. Immunofluorescence was performed using standard procedures with the following antibodies: mouse anti-Aurora B (BD, 1 : 100); mouse anti-H3 phospho-serine 10 (Upstate, 1 : 100); rabbit anti-H3 trimethyl-lysine 9 (Abcam, 1 : 100); and mouse anti-human CenpA (Abcam, 1 : 100). Fluorescence in situ hybridization (FISH) was carried out as described in Moralli and Monaco, 2009.

2.5. Noncoding RNA Expression Analysis. At 72 hours from transfection, total RNA was extracted from each cell line using the RNeasy kit (Qiagen), following the manufacturer's instructions. The RNA was treated with DNAse I (Qiagen) to remove contaminating DNA and reverse-transcribed into

cDNA, using the RETROScript system (Ambion), with random decamer primers.

The quantification of 17 alpha overexpression in transformed cells was conducted by real-time PCR, using the PerfeCta SYBR Green Mix (Quanta Biosciences) on an iCycler machine (Bio-Rad) with the following primers: 17αSat6F TTGTGGTTTGTGGTGGAAAA and 17αSat6R CTCAAAGCGCTCCAAATCTC, and compared to that of a gene homogenously expressed in all cells (CENP B).

2.6. Live Cell Imaging. HT1080 cells were transfected with pH2BmCherry. Selection was applied with G418 at 300 μg/ml, and a population stably expressing the transgene was recovered. The HT1080-H2BmCherry cells were transfected as outlined above with either pI-17a or pIneo2. After 48 hours, the chromosome segregation was analyzed by live cell imaging using a Zeiss LSM 510 confocal microscope for 48 hours. The multitracking function was used to avoid the bleed-through effect. Appropriate z-direction at 1-2 μm for ten sections was captured on 63x oil objective for every 20 minutes for at least 12 hours.

3. Results

3.1. Noncoding Repetitive Satellite DNA-Expressing Vectors. To test the effect of centromere satellite expression on chromosome segregation, we constructed a series of expression vectors on an identical plasmid backbone, pIRESneo2 (pIneo2). The expression of all satellite inserts was initiated by the CMV promoter and the IRES sequence for the neo gene. Four of the vectors carried human or murine centromeric DNA: pI-17α (containing 2.7 kb of human α satellite DNA from chromosome 17), pI21α (containing 1.4 kb of α satellite DNA from chromosome 21), pIYα (containing 0.8 kb of α satellite DNA from chromosome Y), and pI-Minor (carrying 1.7 kb of mouse minor centromeric satellite). As control experiments, two more vectors containing noncentromeric satellite sequences were assembled: pIDYZ1 (carrying 1.1 kb of DYZ1 satellite from the human Y chromosome long arm) and pI-Major (containing 3.2 kb of mouse pericentromeric major satellite). The vectors are shown in Figure 1(a).

3.2. Expression of Centromeric Sequences Induces Mis-Segregation in Anaphase. The human (HUES-10, HT1080) and murine (E14TG2A) cells were transfected with the respective satellite expression constructs outlined above and the insert-less pIneo2 vector in a parallel control experiment. After 72 hours posttransfection, the cells were fixed and one hundred metaphase and anaphase cells on each slide were scored for segregation errors. The presence of delayed chromosome congression was scored as a metaphase error, while the presence of anaphase bridges or lagging chromosomes was scored as an anaphase error. At least three independent experiments were repeated for each vector and cell line, and the number of segregation errors observed for each satellite was compared to that of cells treated with the insert-less pISneo2 vector. The statistical significance of the differences was determined using the Student t-test for independent samples. The results are showed in Table 1 and Figures 1(b) and 1(c).

We further focussed on investigating the pI-17α-transfected cells. Real-time PCR was performed to confirm 17α RNA expression level in three different replicate experiments. On average, following transduction with pI-17α, we found that the centromeric satellites were transcribed at levels comparable to the single-copy control gene. The pI-17α expression levels correlated positively with the frequency of anaphase aberrations ($p = 0.005$): the higher the levels of the centromeric noncoding RNAs, the higher the percentage of cells that showed bridges and/or lagging chromosomes.

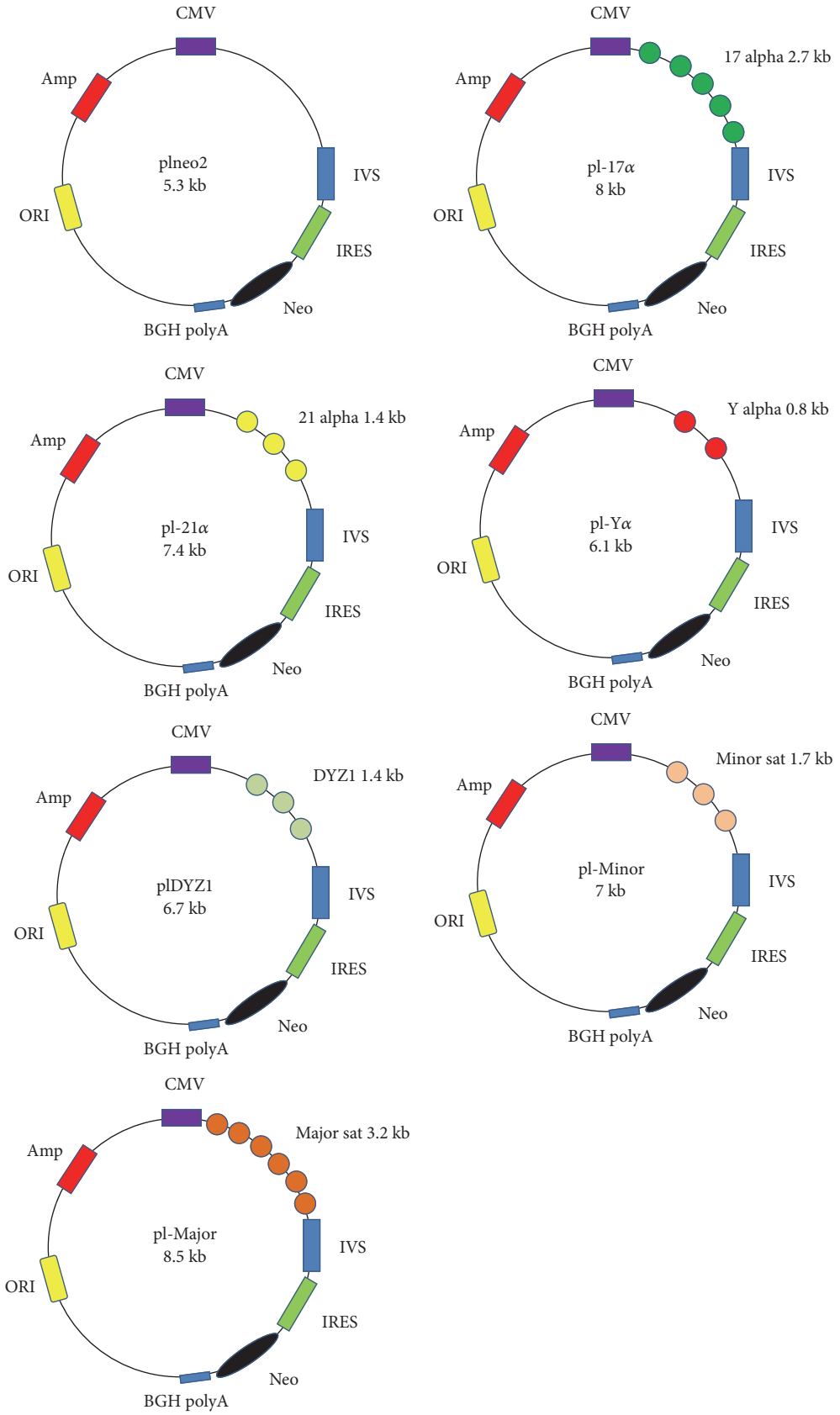
In HUES-10 and HT10180 cells, the overexpression of the 17α, 21α, and Yα human centromeric vectors did not affect the number of chromosomes involved in metaphase congression delay or the number of multipolar spindle cells (data not shown). However, approximately 20% of the anaphase cells analyzed after transfection with each chromosome α vector contained bridging or lagging chromosomes in HT1080 and HUES-10 cells (Figures 1(b) and 1(c)). No significant differences were observed between 17α, 21α, and Yα, in either HT1080 or HUES-10. The overexpression of noncentromeric satellite DYZ1 and pIneo2 vectors had no apparent effect on chromosome behaviour, in either metaphase or anaphase cells. The expression of mouse centromeric minor and major satellite DNA showed no significant effect on either human cell line (Table 1).

To determine if the chromosome 17 was preferentially involved in segregation errors when the 17α satellite was overexpressed, we conducted FISH in HT1080 cells, following transduction with pI-17α or control vectors, using a chromosome 17 whole-chromosome paint. No difference in chromosome 17 segregation efficiency was detected between cells transformed with pI-17α and pIneo2 (data not shown).

In mouse ES cells, none of the human satellites had any detectable effect on murine chromosome segregation. Overexpression of murine minor but not of major satellite DNA resulted in anaphase segregation errors (data no shown) compared to that of the pIneo2 control.

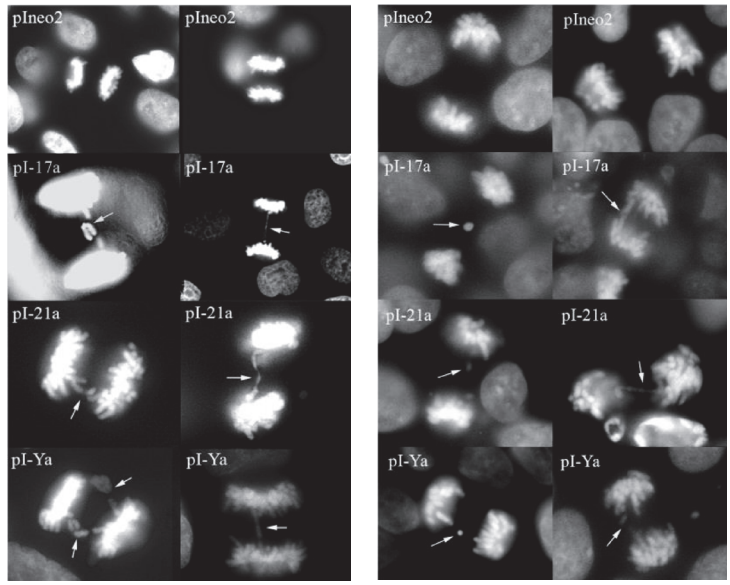
3.3. Immunohistostaining. The effect of centromeric ncRNAs on chromosome structure and segregation was analyzed by staining with specific antibodies for proteins or histone modifications involved in the kinetochore formation and the control of cell cycle progression. The localization of Aurora-B, CENP-C, CENP-A, histone H3 phosphorylated in serine 10, and H3 trimethylated in lysine 9 was investigated in HT1080 cells expressing the 17α and pIRESneo vector DNA. No difference was observed in localization or abundance of each protein in HT1080 cells overexpressing 17α and pIRESneo2 when compared to that in untreated cells (data not shown).

3.4. Live Cell Imaging on 17α-Expressing HT1080 Cells. To visualize the chromosomal segregation events, HT1080 cells were initially transfected with a construct expressing a fusion gene between the H2B histone and the mCherry protein, which when incorporated into the chromatin rendered the chromosome a bright fluorescent red. The H2B-mCherry



(a)

Figure 1: Continued.

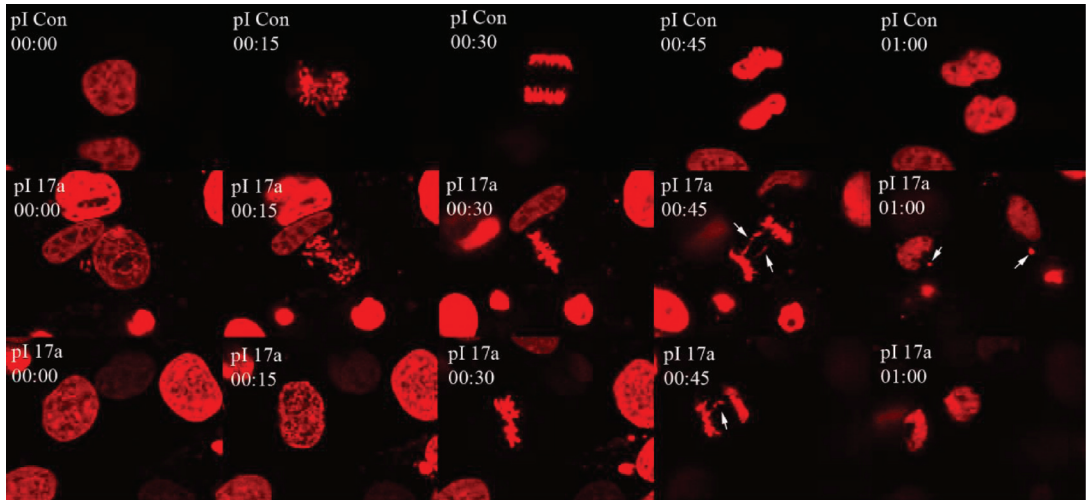


(b) (c)

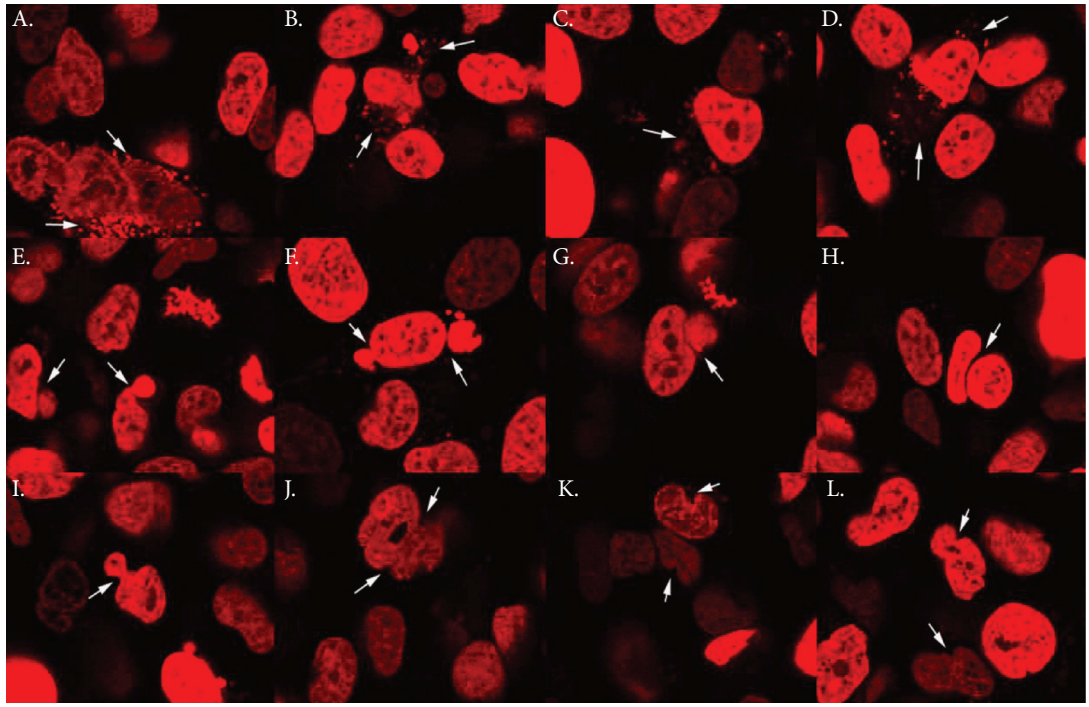
Figure 1: (a) Schematic vector maps. (b, c) Expression of 17α, 21α, and Yα induced mis-segregation in HT1080 (b) and HUES-10 (c) cells (white arrows).

Table 1: The effects of noncoding centromeric RNAs on anaphase abnormalities.

Expression vectors and cell types	Anaphase abnormalities (%); number of error/total events	Significant level	Standard deviation and standard error
	HT 1080 cells		
Control pIneo2	9.7 (136/1400)	NA	3.36/0.93
pI-17α	21.3 (170/800)	<0.0001	7.11/2.51
pI-21α	21.3 (128/600)	<0.0001	5.17/2.11
pI-Yα	25.3 (76/300)	<0.0001	1.15/0.66
pI-DYZ1	7.5 (90/1200)	Not significant	2.90/0.83
pI-Major	10.6 (74/700)	Not significant	3.34/1.26
pI-Minor	13.6 (95/700)	Not significant	3.47/1.37
	HUES-10 cells		
Control pIneo2	5.0 (15/300)	NA	4.58/2.65
pI-17α	18.7 (56/300)	<0.02	6.51/3.76
pI-21α	19.7 (59/300)	<0.029	8.5/4.91
pI-Yα	16.3 (49/300)	<0.014	1.15/0.67
pI-DYZ1	6.7 (20/300)	Not significant	3.79/2.19
pI-Major	6.3 (19/300)	Not significant	4.93/2.85
pI-Minor	5.7 (17/300)	Not significant	7.23/4.18
	mES cells		
Control pIneo2	4.7 (14/300)	NA	2.52/1.45
pI-17α	6.0 (18/300)	Not significant	2.00/1.15
pI-21α	6.0 (18/300)	Not significant	2.00/1.15
pI-Yα	7.3 (22/300)	Not significant	0.58/0.33
pI-DYZ1	6.0 (18/300)	Not significant	3.61/2.08
pI-Major	7.3 (22/300)	Not significant	2.89/1.67
pI-Minor	14.3 (43/300)	<0.025	5.51/3.18



(a)



(b)

Figure 2: Live cell imaging on 17α-expressing H2B-mCherry-HT1080 cells. The pIneo2 controls did not show any mis-segregation ((a), top, left to right). Expression of 17α satellite in HT1080 induced mis-segregation events ((a), middle and bottom, left to right). Time lapse images were taken every 15-minute interval, and the image prior to mitosis was set at time zero. There were several interesting phenotypic effects discovered in 17α-expressing HT1080 cells, stars ((b), A–D), binucleated cells (E–H), and polylobed nuclei (I–L).

HT1080 cells were then transfected with pI-17α and pIneo2 as a control to observe chromosome segregation. Seventy-two hours after transfection, the cells were continuously analyzed by live cell imaging with a confocal microscope for 48 hours. In HT1080 cells transfected with pI-17α, 78 mitotic cells were captured and 33 (42.3%) showed bridging or lagging chromosomes in anaphase (Figure 2). In contrast, none of the H2B-mCHerry HT1080 cells transfected with pIneo2 showed bridging or lagging chromosomes in anaphase (data not shown).

Table 2: The effects of alpha 17 satellite expression in the HT1080 cell.

Alpha 17 satellite expression in HT1080	
Types of abnormalities	% of abnormality
Anaphase abnormality	42.3
Delayed metaphase	9
Polylobed nuclei	23.2
Binuclei	13.8
Stars	39.7

The time lapse analysis clearly showed the presence of a strong red signal, scattered around the nuclei in a "star" phenotype. Nearly 40% of cells observed displayed this phenotype, while 14% of cells appeared binucleated, and 23% of cells had polylobed nuclei (Figure 2 and Table 2).

4. Discussion

In this report, we showed that overexpression of centromeric sequences from different suprachromosomal families induced a similar effect on chromosome segregation in both human stem (HUES-10) and fibrosarcoma (HT1080) cells. The 21α array includes a dimeric high-order repeat (HOR) belonging to the group of suprachromosomal families 1, 2, and 5; the 17α array has a pentameric HOR and belongs to suprachromosomal family 3, and the Yα array lacks a definable monomeric HOR and belongs to family 4 [15]. All of the alphoid suprachromosomal families except for family 4 contain the CENP-B box, a 17 bp motif that serves as a binding site for the centromeric protein B (CENP-B). However, overexpression of Yα in HUES-10 and HT1080 showed the same effect on chromosome segregation in both cell lines. These experiments clearly demonstrated that all human centromeric sequences tested affected segregation events in human tumour-derived and normal cells. This work is similar to a report by Bouzinba-Segard et al., whereby mouse minor satellite RNA expression and accumulation impaired mouse centromeric architecture and function [6].

Wong et al. recently showed that centromeric alphoid RNA is a key component for the assembly of nucleoproteins at the centromere and nucleolus [4], and this process may be disrupted from overexpression of ncRNAs. A more recent report showed that knocking down alpha satellite expression impaired chromosome segregation [16]. These findings are in line with our results, showing that noncoding satellite RNAs play an intriguing role to allow proper segregation to occur. It has been reported by Frescas et al. that KDM2A plays an important role in repressing centromeric satellite repeats; however, the specificity of the repression on chromosomal centromeric repeats was not further narrowed down [17].

Live cell imaging using HT1080-H2B-mCherry cells indicated that the expression of 17α RNA promoted missegregation in anaphase in real time detected with fluorescent histone H2B. Several phenotypic morphologies were observed. The cells overexpressing 17α RNA also revealed a high frequency of several other phenotypic events including binucleated (13.8%) and polylobed nuclei (23.2%) and "stars" (39.7%). Further work will be required to colocalize the noncoding RNA with the affected nuclei. The observations imply that centromeric RNA may be essential to maintain correct progression through mitosis through yet unclear mechanisms. Aberrant nuclear morphology is a feature associated with many forms of cancer cells and their subtypes [18, 19], and it has been speculated that nuclear morphological discrepancies may result from genomic instability.

There are growing evidences that various proteins interact with ncRNAs including CENP-A [20], polymerase II [9], heat shock protein [21], and aurora B [22], for their regulatory effects on chromosomal function and stability. However, none of the previous studies analyzed the effect of chromosome-specific repeats. RNA transcripts from centromeric and pericentromeric repetitive sequences have been identified in different organisms from yeast [23] to human [24], but the underlying mechanism has never been identified. Recent studies showed that alpha satellite nascent RNA is involved in heterochromatin modification. In addition, noncoding centromeric RNAs closely acted on the chromatin condensation or decondensation levels through the transcription of ncRNAs [25]; it is possible that our findings in this study are caused by the disruption of this finely tuned balance. The housekeeping expression level of ncRNA has never been studied, but it was suggested that the expression level could be affected by external factors such as temperature and chemical stress. Perturbation of the chromatin status elicited by ncRNAs is not surprising when related to the pathological status of the organism, as there were reports showing that elevation of ncRNA is involved in several types of cancers [8, 26–29].

Our study shows that the chromosome-specific alpha satellite RNAs affect the segregation of all chromosomes, as well as nuclear morphology. Further experiments are required to understand how centromeric ncRNAs affect chromosome segregation. This would include investigating the relationship between the ncRNA expression level and the severity of segregation errors incurred and understanding the downstream mechanism involved in centromeric RNA effects. However, our data suggest that evaluation of the levels of centromeric RNA expression and specific alterations in nuclear cell morphology could represent a useful cancer biomarker for some tumours.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] B. Grimes and H. Cooke, "Engineering mammalian chromosomes," *Human Molecular Genetics*, vol. 7, no. 10, pp. 1635–1640, 1998.

[2] H. F. Willard, "Evolution of alpha satellite," *Current Opinion in Genetics & Development*, vol. 1, no. 4, pp. 509–514, 1991.

[3] T. Fukagawa and W. C. Earnshaw, "The centromere: chromatin foundation for the kinetochore machinery," *Developmental Cell*, vol. 30, no. 5, pp. 496–508, 2014.

[4] L. H. Wong, K. H. Brettingham-Moore, L. Chan et al., "Centromere RNA is a key component for the assembly of nucleoproteins at the nucleolus and centromere," *Genome Research*, vol. 17, no. 8, pp. 1146–1160, 2007.

[5] F. Ferri, H. Bouzinba-Segard, G. Velasco, F. Hube, and C. Francastel, "Non-coding murine centromeric transcripts associate with and potentiate Aurora B kinase," *Nucleic Acids Research*, vol. 37, no. 15, pp. 5071–5080, 2009.

[6] H. Bouzinba-Segard, A. Guais, and C. Francastel, "Accumulation of small murine minor satellite transcripts leads to impaired centromeric architecture and function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 23, pp. 8709–8714, 2006.

[7] A. Eymery, B. Horard, M. El Atifi-Borel et al., "A transcriptomic analysis of human centromeric and pericentric sequences in normal and tumor cells," *Nucleic Acids Research*, vol. 37, no. 19, pp. 6340–6354, 2009.

[8] D. T. Ting, D. Lipson, S. Paul et al., "Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers," *Science*, vol. 331, no. 6017, pp. 593–596, 2011.

[9] F. L. Chan, O. J. Marshall, R. Saffery et al., "Active transcription and essential role of RNA polymerase II at the centromere during mitosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 6, pp. 1979–1984, 2012.

[10] L. E. Hall, S. E. Mitchell, and R. J. O'Neill, "Pericentric and centromeric transcription: a perfect balance required," *Chromosome Research*, vol. 20, no. 5, pp. 535–546, 2012.

[11] D. L. Grady, R. L. Ratliff, D. L. Robinson, E. C. McCanlies, J. Meyne, and R. K. Moyzis, "Highly conserved repetitive DNA sequences are present at human centromeres," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 5, pp. 1695–1699, 1992.

[12] K. H. Choo, B. Vissel, A. Nagy, E. Earle, and P. Kalitsis, "A survey of the genomic distribution of alpha satellite DNA on all the human chromosomes, and derivation of a new consensus sequence," *Nucleic Acids Research*, vol. 19, no. 6, pp. 1179–1182, 1991.

[13] A. Joseph, A. R. Mitchell, and O. J. Miller, "The organization of the mouse satellite DNA at centromeres," *Experimental Cell Research*, vol. 183, no. 2, pp. 494–500, 1989.

[14] H. Masumoto, H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki, "A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite," *The Journal of Cell Biology*, vol. 109, no. 5, pp. 1963–1973, 1989.

[15] A. L. Jorgensen, "Alphoid repetitive DNA in human chromosomes," *Danish Medical Bulletin*, vol. 44, no. 5, pp. 522–534, 1997.

[16] T. Ideue, Y. Cho, K. Nishimura, and T. Tani, "Involvement of satellite I noncoding RNA in regulation of chromosome segregation," *Genes to Cells*, vol. 19, no. 6, pp. 528–538, 2014.

[17] D. Frescas, D. Guardavaccaro, S. M. Kuchay et al., "KDM2A represses transcription of centromeric satellite repeats and maintains the heterochromatic state," *Cell Cycle*, vol. 7, no. 22, pp. 3539–3547, 2008.

[18] T. Kudo, S. E. Kudo, Y. Mori et al., "Classification of nuclear morphology in endocytoscopy of colorectal neoplasms," *Gastrointestinal Endoscopy*, vol. 85, no. 3, pp. 628–638, 2017.

[19] K. Magnusson, G. Gremel, L. Ryden et al., "ANLN is a prognostic biomarker independent of Ki-67 and essential for cell cycle progression in primary breast cancer," *BMC Cancer*, vol. 16, no. 1, p. 904, 2016.

[20] D. Quenet and Y. Dalal, "A long non-coding RNA is required for targeting centromeric protein A to the human centromere," *eLife*, vol. 3, article e03254, 2014.

[21] R. Valgardsdottir, I. Chiodi, M. Giordano et al., "Transcription of satellite III non-coding RNAs is a general stress response in human cells," *Nucleic Acids Research*, vol. 36, no. 2, pp. 423–434, 2008.

[22] J. P. Mallm and K. Rippe, "Aurora kinase B regulates telomerase activity via a centromeric RNA in stem cells," *Cell Reports*, vol. 11, no. 10, pp. 1667–1678, 2015.

[23] K. Ohkuni and K. Kitagawa, "Endogenous transcription at the centromere facilitates centromere activity in budding yeast," *Current Biology*, vol. 21, no. 20, pp. 1695–1703, 2011.

[24] R. Saffery, H. Sumer, S. Hassan et al., "Transcription within a functional human centromere," *Molecular Cell*, vol. 12, no. 2, pp. 509–516, 2003.

[25] Z. Pezer and D. Ugarkovic, "Role of non-coding RNA and heterochromatin in aneuploidy and cancer," *Seminars in Cancer Biology*, vol. 18, no. 2, pp. 123–130, 2008.

[26] V. Alexiadis, M. E. Ballestas, C. Sanchez et al., "RNAPol-ChIP analysis of transcription from FSHD-linked tandem repeats and satellite DNA," *Biochimica et Biophysica Acta*, vol. 1769, no. 1, pp. 29–40, 2007.

[27] N. I. Enukashvily, R. Donev, I. S. Waisertreiger, and O. I. Podgornaya, "Human chromosome 1 satellite 3 DNA is decondensed, demethylated and transcribed in senescent cells and in A431 epithelial carcinoma cells," *Cytogenetic and Genome Research*, vol. 118, no. 1, pp. 42–54, 2007.

[28] D. K. Shumaker, T. Dechat, A. Kohlmaier et al., "Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 23, pp. 8703–8708, 2006.

[29] Q. Zhu, G. M. Pao, A. M. Huynh et al., "BRCA1 tumour suppression occurs via heterochromatin-mediated silencing," *Nature*, vol. 477, no. 7363, pp. 179–184, 2011.

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Research Article

Association of Single-Nucleotide Polymorphisms in DC-SIGN with Nasopharyngeal Carcinoma Susceptibility

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The aim of this study was to explore potential relationships of four single-nucleotide polymorphisms (SNPs) in the gene encoding dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) with risk of nasopharyngeal carcinoma (NPC). The DC-SIGN SNPs rs7252229, rs4804803, rs2287886, and rs735240 were genotyped in 477 unrelated NPC patients and 561 cancer-free controls. At rs7252229, risk of NPC was significantly lower in individuals with GC (odds ratio [OR] 0.076, 95% confidence interval [CI] 0.008–0.690), GG (OR 0.056, 95%CI 0.006–0.487), or GC + GG (OR 0.059, 95%CI 0.007–0.515) than in individuals with the CC genotype, after adjusting for age, gender, smoking history, and EBV-VCA-IgA status. At rs4804803, risk of NPC was significantly higher in individuals with the genotype GG than in those with the genotype AA (adjusted OR 9.038, 95%CI 1.708–47.822). At rs735240, risk of NPC did not change significantly with genotypes AG, GG, or AG + GG after adjusting for age, gender, and smoking history. However, when data were also adjusted for EBV-VCA-IgA status, three genotypes emerged as associated with significantly higher risk of NPC than the AA genotype: AG (OR 2.976, 95%CI 1.123–7.888), GG (OR 3.314, 95%CI 1.274–8.622), or GG + AG (OR 3.191, 95%CI 1.237–8.230). Our results suggest that DC-SIGN SNPs rs7252229, rs4804803, and rs735240 may influence NPC risk in the Chinese population. The mechanisms mediating this risk require a further study.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern China, especially Guangdong and Guangxi provinces. In these endemic areas, annual NPC incidence reaches 20–30 per 100,000 [1]. The primary risk factors for NPC include Epstein-Barr virus (EBV) infection, environmental carcinogens, and certain ethnic backgrounds [2].

EBV infection is strongly associated with NPC pathogenesis [3], and approximately 98% of all NPC cases are EBV-related [4]. EBV may help drive NPC by encoding several oncogenic proteins, such as LMP1, which transform infected epithelial cells. In addition, the virus helps transformed cells evade host immune responses [5, 6]. One protein involved in this immune evasion may be dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), a type II membrane protein of the C-type lectin receptor superfamily [7] that is encoded by CD209 on chromosome 19p13.3. Expressed mainly on the surface of immature dendritic cells (DCs), macrophages, and B lymphocytes, DC-SIGN recognizes and induces interactions with many pathogens, including bacteria, virus, and parasites [8–13]. DC-SIGN on immature DCs can bind carcinoembryonic antigen (CEA), inhibiting DC maturation and thereby inducing tolerance of tumor cells [9]. The SKBR3

breast carcinoma cell line can interact with DC-SIGN on macrophages to induce interleukin-10 secretion, contributing to an immunosuppressive environment [14].

Several single-nucleotide polymorphisms (SNPs) in the DC-SIGN gene have been associated with elevated risk of human diseases such as tuberculosis, dengue, AIDS, and cancer. For example, the SNP rs2287886 in the area of the promoter and SNP rs7248637 in the 3′-untranslated region may be associated with susceptibility to colorectal carcinoma [15]. The SNPs rs2287886, rs735240, and rs735239 correlated with NPC risk in a Cantonese population from Guangdong [16], while rs4804800 and rs7248637 correlated with risk in a North African population [10].

In the present study, we investigated the possible genetic associations of the DC-SIGN SNPs with NPC risk in a Chinese population in the NPC-endemic Guangxi province of China.

2. Methods

2.1. Study Participants. The study was approved by the Ethics Committee of Guangxi Medical University. Unrelated NPC patients who were diagnosed with NPC and treated between July 2012 and June 2015 at the Affiliated Tumor Hospital of Guangxi Medical University (Guangxi, China) were enrolled. In addition, 561 healthy controls were recruited among those undergoing routine physical examinations at the Affiliated Tumor Hospital and the First Affiliated Hospital of Guangxi Medical University. The selection criteria for the healthy controls included the following: (1) no individual history of malignant tumors, (2) undergoing a health examination during the same enrollment period as the NPC cases, and (3) a local resident of Guangxi province. All study participants provided written informed consent.

2.2. Blood Collection. Peripheral venous blood (3 mL) was drawn from all subjects into EDTA-containing tubes, and genomic DNA was isolated using the TGuide Blood Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. DNA quality was evaluated using agarose gel electrophoresis. Samples of verifiable quality were stored at −20°C.

2.3. Selection of SNPs. A total of 4 SNPs (rs7252229, rs2287886, rs4804803, and rs735240) within DC-SIGN were selected to the following analyses based on the following selection strategy: (1) Using the 1000 Genomes Browser (http://phase3browser.1000genomes.org/index.html) and the NCBI database (http://www.ncbi.nlm.nih.gov), SNP files of DC-SIGN were obtained for the Beijing Han Chinese (CHB) population. (2) Using Haploview 4.2 software, the SNP files were analyzed and the tagging SNPs which met the below criteria were selected: (i) minimum allele frequency (MAF) of greater than 5% in the CHB population; (ii) location within the coding region or the promoter region or the 3′- or 5′-untranslated regions (UTRs); and (iii) no strong linkage disequilibrium (LD; $r^2 < 0.80$) with any other selected SNPs. (3) The potential function of the tagging SNPs was analyzed using SNP function prediction in the website http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm. (4) We also forced the inclusion of some SNPs which did not fulfill the above selection criteria, for their functionality has been indicated in published studies. (5) Compatibility of selected SNPs through the above steps with the platform of MALDI-TOF mass spectrometry-based genotyping was analyzed by the BGI company (Beijing, China), which provided the SNP genotyping support. And the SNPs which were not technically compatible with the genotyping platform were excluded from the following formal genotyping.

2.4. Mass Spectrometry-Based Genotyping. The DC-SIGN SNPs rs7252229, rs4804803, rs2287886, and rs735240 were genotyped in all subjects as described [17] using allele-specific matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, with support from BGI (Beijing, China). Fragments spanning the SNPs were amplified by PCR using primers designed with MassARRAY Assay Design 3.1 Software (Sequenom, San Diego, CA, USA). Amplifications were conducted in a 384-well ABI Veriti PCR System (Applied Biosystems) following the manufacturer's instructions. Amplification reactions (5 μL) contained 4 μL Master Mix and 1 μL DNA (20 ng/μL), and reaction conditions were as follows: 94°C for 5 min, followed by 45 cycles of 94°C for 20 sec, 56°C for 30 sec, and 72°C for 1 min. Alleles were analyzed using MassARRAY TYPER 4.0 software (Sequenom). Successful genotyping rates were 99.8% (560/561) in the control group for rs7252229, rs4804803, and rs735240, respectively, and were 99.8% (476/477) in the NPC group and 99.6% (559/561) in the control group for rs2287886.

2.5. Statistical Analysis. Data were analyzed using SPSS 17.0 (IBM, Armonk, NY, USA), and the threshold for significance was defined as two-sided $P < 0.05$. NPC patients and healthy controls were compared in terms of gender composition, smoking history, and the presence of immunoglobulin A against EBV capsid antigen (EBV-VCA-IgA) using the chi-squared test. The ages of patients and controls were compared using an unpaired *t*-test. Genotype distributions in healthy controls were compared against the predictions of Hardy-Weinberg equilibrium (HWE) using the Pearson chi-squared test. Genotype frequencies were compared between patients and controls using the chi-squared test. Possible associations between DC-SIGN SNP genotypes and risk of NPC were identified using logistic regression; odds ratios (ORs) with 95% confidence intervals (CIs) were calculated when appropriate. Unconditional multivariate logistic regression was used to calculate adjusted ORs and 95%CIs after adjusting for age, gender, smoking history, and EBV-VCA-IgA status, unless otherwise noted. The goal was to eliminate possible effects of these confounding factors in order to isolate the effects of each SNP on NPC risk.

3. Results

3.1. Characteristics of Patients and Controls. A total of 477 NPC patients and 561 healthy controls were included in our study. While the two groups were similar in

age ($P = 0.056$), they differed significantly in gender composition ($P < 0.001$), smoking history ($P < 0.001$), and EBV-VCA-IgA status ($P < 0.001$; Table 1). Frequencies of each SNP genotype were in agreement with HWE in the control population: rs7252229, $P = 0.307$; rs4804803, $P = 0.533$; rs2287886, $P = 0.331$; and rs735240, $P = 0.937$.

3.2. Correlation of rs7252229 Polymorphism with Risk of NPC. Frequency of the genotype GG + GC at rs7252229 was significantly lower in NPC patients than in healthy controls (0.18% versus 1.68%), corresponding to an unadjusted OR of 0.105 (95%CI 0.013–0.842) and an adjusted OR of 0.059 (95%CI 0.007–0.515; Table 2). The genotypes GC and GG were also associated with lower risk than the genotype CC: the respective unadjusted ORs were 0.111 (95%CI 0.014–0.914) and 0.104 (95%CI 0.013–0.834); the corresponding adjusted ORs were 0.076 (0.008–0.690) and 0.056 (95%CI 0.006–0.488).

3.3. Correlation of rs2287886 Polymorphism with Risk of NPC. Relative to individuals with the genotype AA, individuals with the genotype AG or GG were at similar risk of NPC based on unadjusted and adjusted OR calculations (Table 3).

3.4. Correlation of rs4804803 Polymorphism with Risk of NPC. Individuals with the genotype GG were at significantly higher risk of NPC than those with the genotype AA (Table 4). The unadjusted OR was 4.800 (95%CI 1.014–22.732) and the adjusted OR was 9.038 (95%CI 1.708–47.822).

3.5. Correlation of rs735240 Polymorphism with Risk of NPC. Frequencies of genotypes AA, AG, and GG did not differ significantly between patients and controls before or after adjusting for age, gender, and smoking history. Interestingly, after controlling for EBV-VCA-IgA status, the genotypes GG, AG, and GG + AG emerged as conferring higher risk of NPC, with respective ORs of 2.976 (95%CI 1.123–7.888), 3.314 (95%CI 1.274–8.622), and 3.191 (95%CI 1.237–8.230) (Table 5). Next, we compared genotype frequencies between patients and controls in separate subgroups among all subjects negative for EBV-VCA-IgA and among all subjects positive for EBV-VCA-IgA. Among those negative for EBV-VCA-IgA, the genotypes GG, AG, and GG + AG were significantly more frequent among patients than controls, with respective ORs of 8.797 (95%CI 1.175–65.838), 2.077 (95%CI 1.055–4.087), and 8.710 (95%CI 1.168–64.940). Among subjects positive for EBV-VCA-IgA, in contrast, genotype frequencies were similar between patients and controls.

Table 1: Clinical characteristic of Chinese patients with NPC and cancer-free controls.

Subgroup	Cases	Controls	χ^2/t	P
N	477	561		
Gender, n			42.121	<0.001[a]
Male	365	322		
Female	112	239		
Age, yr	46.68 ± 11.60	48.17 ± 13.53	1.912	0.056[b]
EBV-VCA-IgA status, n			197.811	<0.001[a]
Positive		15		
Negative	314	546		
Smoking history, n	163		21.883	<0.001[a]
Yes	163	119		
No	314	442		

[a]Chi-squared test; [b]two-sample t-test. EBV-VCA-IgA: immunoglobulin A against Epstein-Barr virus capsid antigen; NPC: nasopharyngeal carcinoma; OR: odds ratio.

4. Discussion

Our data showed significantly lower frequency of the GC + GG genotypes at rs7252229 in NPC patients than in healthy controls, suggesting that the genotype CC increases risk of NPC. This appears to be the first report linking rs7252229 with risk of malignant disease. Another study linked the C allele at rs7252229 with elevated risk of invasive pulmonary aspergillosis [11]. How rs7252229 may contribute to NPC pathogenesis is unclear. Since rs7252229 lies within an intron in the DC-SIGN gene and it is predicted to be a binding site for some transcripts (based on http://snpinfo.niehs.nih.gov/cgi-bin/snpinfo/snpfunc.cgi), we predict that the genotype CC at rs7252229 may affect DC-SIGN expression and thereby overall activity. It is also possible that rs7252229 polymorphism affects humoral and cell-mediated immunity: in a study of healthy school children and young adults who received the measles vaccine, the genotype CC was associated with higher specific neutralizing antibody titers than the genotype GG among African-Americans, while the genotype CC was associated with lower interferon-γ levels than genotypes GG and GC among Caucasians [12]. Thus, it is plausible that this SNP might be associated with immunological abnormality in NPC patients as well as immune escape by EBV-infected NPC cells. This should be investigated in future studies.

Our data showed a significantly higher frequency of the genotype GG at rs4804803 in patients than in controls, indicating that this DC-SIGN SNP is associated with elevated NPC risk. Our results are inconsistent with those of studies in a Cantonese population [16] and a North African population [10] that found no relation between this SNP and NPC risk. Two possible explanations for this discrepancy are that (1) differences in genetic background and linkage disequilibrium between our population of mostly Zhuang minority subjects and the Cantonese subjects in the previous study [16] may mean different effects of DC-SIGN polymorphism on NPC risk and/or (2) DC-SIGN alleles are present at different frequencies across populations. Indeed, the frequency of the G allele in our control group was 8% (82/1038), much lower than the frequencies of approximately 26% reported for Caucasian and North African populations [10, 13]. How the SNP rs4804803 may influence NPC risk is uncertain. It is located only 214 bp upstream from the major Sp1-like binding region. We speculate that polymorphism in this

Table 2: Frequencies of genotypes at DC-SIGN rs7252229 in NPC patients and healthy controls.

Genotype	Cases	Controls	Unadjusted OR (95%CI)	P	Adjusted OR* (95%CI)	Adjusted P	P_{HWE}
rs7252229							0.307
CC	8	1	1.000		1.000		
GC	65	73	0.111 (0.014–0.914)	0.041	0.076 (0.008–0.690)	0.022	
GG	404	486	0.104 (0.013–0.834)	0.033	0.056 (0.006–0.487)	0.009	
GC + GG	469	559	0.105 (0.013–0.842)	0.034	0.059 (0.007–0.515)	0.010	

*Calculated using multiple logistic regression after controlling for age, sex, smoking history, and EBV infection factors. CI: confidence interval; DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; HWE: Hardy-Weinberg equilibrium; NPC: nasopharyngeal carcinoma; OR: odds ratio.

Table 3: Frequencies of genotypes at DC-SIGN rs2287886 in NPC patients and healthy controls.

Genotype	Cases	Controls	Unadjusted OR (95%CI)	P	Adjusted OR* (95%CI)	Adjusted P	P_{HWE}
rs2287886							0.331
AA	236	294	1.000		1.000		
AG	202	229	1.099 (0.851–1.418)	0.469	1.174 (0.778–1.772)	0.444	
GG	38	36	1.315 (0.808–2.140)	0.270	0.819 (0.345–1.94)	0.651	

*Calculated using multiple logistic regression after controlling for age, sex, smoking history, and EBV infection factors. CI: confidence interval; DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; HWE: Hardy-Weinberg equilibrium; NPC: nasopharyngeal carcinoma; OR: odds ratio.

Table 4: Frequencies of genotypes at DC-SIGN rs4804803 in NPC patients and healthy controls.

Genotype	Cases	Controls	Unadjusted OR (95%CI)	P	Adjusted OR* (95%CI)	Adjusted P	P_{HWE}
rs4804803							0.533
AA	400	480	1.000		1.000		
AG	69	78	1.062 (0.748–1.506)	0.738	1.269 (0.803–2.005)	0.307	
GG	8	2	4.800 (1.014–22.732)	0.048	9.038 (1.708–47.822)	0.010	
GG + AG	77	80	1.155 (0.822–1.623)	0.406	1.447 (0.934–2.241)	0.098	

*Calculated using multiple logistic regression after controlling for age, sex, smoking history, and EBV-VCA-IgA status. CI: confidence interval; DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; HWE: Hardy-Weinberg equilibrium; NPC: nasopharyngeal carcinoma; OR: odds ratio.

Table 5: Frequencies of genotypes at DC-SIGN rs735240 in NPC patients and healthy controls.

Genotype	Cases	Controls	Unadjusted OR (95%CI)	P	Adjusted OR*(95%CI)	Adjusted P	Adjusted OR▲ (95%CI)	Adjusted P	P_{HWE}
rs735240									0.937
AA	24	28	1.000		1.000		1.000		
AG	161	193	0.973 (0.543–1.745)	0.927	1.044 (0.574–1.901)	0.887	2.976 (1.123–7.888)	0.028	
GG	292	339	1.005 (0.570–1.772)	0.986	1.077 (0.601–1.927)	0.804	3.314 (1.274–8.622)	0.014	
AG + GG	453	532	0.993 (0.568–1.738)	0.982	1.065 (0.600–1.891)	0.830	3.191 (1.237–8.230)	0.016	

*Calculated using multiple logistic regression after controlling for age, sex, and smoking history; ▲calculated using multiple logistic regression after controlling for age, sex, smoking history, and EBV-VCA-IgA status. CI: confidence interval; DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; HWE: Hardy-Weinberg equilibrium; NPC: nasopharyngeal carcinoma; OR: odds ratio.

region affects DC-SIGN gene transcription and thereby its expression [16].

The SNP rs2287886, located close to the AP-1 binding site, was not associated with NPC risk in our study. While this result is consistent with the findings in a North African population [10], it contrasts with a previous report showing that Guangdong individuals carrying the G allele at rs2287886 were at 1.42-fold higher risk of NPC than those

with the A allele [16]. This discrepancy may reflect differences in genetic background and linkage disequilibrium between our mostly Zhuang minority subjects and the Cantonese subjects in the Guangdong study.

We observed no significant relationship of rs735240 with risk of NPC based on unadjusted ORs or ORs adjusted for age, sex, and smoking history. However, we found that the frequency of the genotype AA was significantly lower in patients than in controls among subjects negative for EBV-VCA-IgA. Thus, our results suggest that the genotype AA may protect against NPC in individuals negative for EBV-related antibodies. This implies that the SNP rs735240 and EBV infection may interact to influence risk of NPC.

How this interaction occurs is unclear. EBV can infect DC-SIGN-positive cells including monocytes, immature DCs, and some macrophages [16, 18, 19]. Polymorphism in the DC-SIGN gene has already been shown to influence how easily DCs that are infected with cytomegalovirus, a herpesvirus with a structure similar to that of EBV: DCs from individuals with the genotype GG at rs735240 and the genotype AA at rs2287886 (GGAA) express higher levels of DC-SIGN and are more efficiently infected by cytomegalovirus than DCs carrying the AAGG genotype [20]. An analogous situation may exist for EBV, since glycoproteins on the viral surface, which are conserved with those on cytomegalovirus [21], appear to bind to DC-SIGN [16, 22, 23] and allow the virus to enter B cells and epithelial cells [24]. This leads us to propose that the genotype AA at rs735240 protects against NPC by reducing DC-SIGN expression, making it more difficult for EBV to infect DCs and nasopharyngeal epithelial cells.

It is noteworthy in our study that the NPC and control group differed significantly in gender composition and smoking status (Table 1). Although these differences suggested that sex and smoking habit might be confounding factors affecting the results, no significant differences in our following analyses were showed in genotype frequencies at rs2887886 and rs4804803 and rs7252229 and rs735240 between males and females or ever-smokers and never-smokers in both NPC and control groups (detail not shown). Thus, it is unlikely that these SNPs vary with gender or smoking status. In any case, we controlled for sex, smoking status, and age by performing adjustment analyses using logistic regression, thereby eliminating or minimizing any possible confounding effects of these factors.

The results of the present study should be interpreted with caution given its important limitations. One is the relatively small sample, which increases the risk of selection bias. Another is our cross-sectional design, which means we cannot exclude the possibility that some of our controls will go on to develop NPC or other malignant tumors.

5. Conclusion

The present study identifies several SNPs in the promoter region of the DC-SIGN gene that may play an important role in NPC pathogenesis. The genotype CC at rs7252229 and the genotype GG at rs4804803 may be associated with elevated NPC risk, while the genotype AA at rs735240 may be associated with decreased NPC risk among individuals negative for EBV-VCA-IgA.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Sisi Li and Zhifang Lu contributed equally to this work.

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References

[1] Y. Xie, Y. Wu, X. Zhou, M. Yao, S. Ning, and Z. Wei, "Association of polymorphisms hOGGI rs1052133 and hMUTYH rs3219472 with risk of nasopharyngeal carcinoma in a Chinese population," *OncoTargets and Therapy*, vol. 9, pp. 755–760, 2016.

[2] A. Edreis, M. A. Mohamed, N. S. Mohamed, and E. E. Siddig, "Molecular detection of Epstein - Barr virus in nasopharyngeal carcinoma among Sudanese population," *Infectious Agents and Cancer*, vol. 11, p. 55, 2016.

[3] A. Hildesheim and C. P. Wang, "Genetic predisposition factors and nasopharyngeal carcinoma risk: a review of epidemiological association studies, 2000-2011: Rosetta stone for NPC: genetics, viral infection, and other environmental factors," *Seminars in Cancer Biology*, vol. 22, no. 2, pp. 107–116, 2012.

[4] S. W. Tsao, C. M. Tsang, K. F. To, and K. W. Lo, "The role of Epstein-Barr virus in epithelial malignancies," *The Journal of Pathology*, vol. 235, no. 2, pp. 323–333, 2015.

[5] Y. Shen, S. Zhang, R. Sun, T. Wu, and J. Qian, "Understanding the interplay between host immunity and Epstein-Barr virus in NPC patients," *Emerging Microbes & Infections*, vol. 4, no. 3, article e20, 2015.

[6] M. E. Ressing, M. van Gent, A. M. Gram, M. J. Hooykaas, S. J. Piersma, and E. J. Wiertz, "Immune evasion by Epstein-Barr virus," *Current Topics in Microbiology and Immunology*, vol. 391, pp. 355–381, 2015.

[7] F. Zhang, S. Ren, and Y. Zuo, "DC-SIGN, DC-SIGNR and LSECtin: C-type lectins for infection," *International Reviews of Immunology*, vol. 33, no. 1, pp. 54–66, 2014.

[8] T. B. Geijtenbeek, D. S. Kwon, R. Torensma et al., "DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells," *Cell*, vol. 100, no. 5, pp. 587–597, 2000.

[9] K. P. van Gisbergen, C. A. Aarnoudse, G. A. Meijer, T. B. Geijtenbeek, and Y. van Kooyk, "Dendritic cells recognize tumor-specific glycosylation of carcinoembryonic antigen on colorectal cancer cells through dendritic cell–specific intercellular adhesion molecule-3–grabbing nonintegrin," *Cancer Reaserch*, vol. 65, no. 13, pp. 5935–5944, 2005.

[10] K. Moumad, J. Lascorz, M. Bevier et al., "Genetic polymorphisms in host innate immune sensor genes and the risk of

nasopharyngeal carcinoma in North Africa," *G3 (Bethesda)*, vol. 3, no. 6, pp. 971–977, 2013.

[11] J. Sainz, C. B. Lupianez, J. Segura-Catena et al., "Dectin-1 and DC-SIGN polymorphisms associated with invasive pulmonary aspergillosis infection," *PloS One*, vol. 7, no. 2, p. e32273, 2012.

[12] I. G. Ovsyannikova, I. H. Haralambieva, R. A. Vierkant, M. M. O'Byrne, R. M. Jacobson, and G. A. Poland, "The association of CD46, SLAM and CD209 cellular receptor gene SNPs with variations in measles vaccine-induced immune responses: a replication study and examination of novel polymorphisms," *Human Heredity*, vol. 72, no. 3, pp. 206–223, 2011.

[13] M. A. Portman, H. W. Wiener, M. Silva, A. Shendre, and S. Shrestha, "DC-SIGN gene promoter variants and IVIG treatment response in Kawasaki disease," *Pediatric Rheumatology*, vol. 11, no. 1, p. 32, 2013.

[14] A. Domínguez-Soto, E. Sierra-Filardi, A. Puig-Kröger et al., "Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10," *The Journal of Immunology*, vol. 186, no. 4, pp. 2192–2200, 2011.

[15] S. Lu, M. Bevier, S. Huhn et al., "Genetic variants in C-type lectin genes are associated with colorectal cancer susceptibility and clinical outcome," *International Journal of Cancer*, vol. 133, no. 10, pp. 2325–2333, 2013.

[16] Y. F. Xu, W. L. Liu, J. Q. Dong et al., "Sequencing of DC-SIGN promoter indicates an association between promoter variation and risk of nasopharyngeal carcinoma in Cantonese," *BMC Medical Genetics*, vol. 11, p. 161, 2010.

[17] E. Schaeffeler, U. M. Zanger, M. Eichelbaum, S. Asante-Poku, J. G. Shin, and M. Schwab, "Highly multiplexed genotyping of thiopurine s-methyltransferase variants using MALDI-TOF mass spectrometry: reliable genotyping in different ethnic groups," *Clinical Chemistry*, vol. 54, no. 10, pp. 1637–1647, 2008.

[18] L. Li, D. Liu, L. Hutt-Fletcher, A. Morgan, M. G. Masucci, and V. Levitsky, "Epstein-Barr virus inhibits the development of dendritic cells by promoting apoptosis of their monocyte precursors in the presence of granulocyte macrophage–colony-stimulating factor and interleukin-4," *Blood*, vol. 99, no. 10, pp. 3725–3734, 2002.

[19] M. Severa, E. Giacomini, V. Gafa et al., "EBV stimulates TLR- and autophagy-dependent pathways and impairs maturation in plasmacytoid dendritic cells: implications for viral immune escape," *European Journal of Immunology*, vol. 43, no. 1, pp. 147–158, 2013.

[20] M. Mezger, M. Steffens, C. Semmler et al., "Investigation of promoter variations in dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (CD209) and their relevance for human cytomegalovirus reactivation and disease after allogeneic stem-cell transplantation," *Clinical Microbiology and Infection*, vol. 14, no. 3, pp. 228–234, 2008.

[21] H. G. Burke and E. E. Heldwein, "Crystal structure of the human cytomegalovirus glycoprotein B," *PLoS Pathogens*, vol. 11, no. 10, article e1005227, 2015.

[22] M. Backovic, R. Longnecker, and T. S. Jardetzky, "Structure of a trimeric variant of the Epstein-Barr virus glycoprotein B," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2880–2885, 2009.

[23] J. J. Reimer, M. Backovic, C. G. Deshpande, T. Jardetzky, and R. Longnecker, "Analysis of Epstein-Barr virus glycoprotein B functional domains via linker insertion mutagenesis," *Journal of Virology*, vol. 83, no. 2, pp. 734–747, 2009.

[24] L. M. Hutt-Fletcher, "Epstein-Barr virus entry," *Journal of Virology*, vol. 81, no. 15, pp. 7825–7832, 2007.

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Review Article

Long Noncoding RNAs as Biomarkers in Cancer

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Long noncoding RNAs (lncRNAs) are a relatively well-characterized class of noncoding RNA (ncRNA) molecules, involved in the regulation of various cell processes, including transcription, intracellular trafficking, and chromosome remodeling. Their deregulation has been associated with the development and progression of various cancer types, the fact which makes them suitable as biomarkers for cancer diagnosis and prognosis. In recent years, detection of cancer-associated lncRNAs in body fluids of cancer patients has proven itself as an especially valuable method to effectively diagnose cancer. Cancer diagnosis and prognosis employing circulating lncRNAs are preferential when compared to classical biopsies of tumor tissues, especially due to their noninvasiveness, and have great potential for routine usage in clinical practice. Thus, this review focuses on summarizing the perspectives of lncRNAs as biomarkers in cancer, based on evaluating their expression profiles determined in body fluids of cancer patients.

1. Introduction

Long noncoding RNAs (lncRNAs) belong to a larger group of noncoding RNAs (ncRNAs) and are generally classified as 200 nt–100 kb long transcripts, lacking the open-reading frame [1, 2]. They are usually transcribed by RNA polymerase II and controlled by the transcriptional activators of the SWI/SNF complex. Most of the generated lncRNA transcripts are usually spliced, capped, and polyadenylated in a similar manner as mRNA molecules [3]. lncRNAs represent a large (>80%) and a very heterogeneous group of ncRNAs, with their expression depending largely on the tissue and cellular context [4–7]. Following the discovery of H19 and XIST lncRNAs in 1990s [8, 9], lncRNA per se was initially regarded as a transcriptional noise with practically no or very little function [10]. However, after being identified as a class of RNA molecules in 2002 [11], studies that followed revealed lncRNA importance and indispensability in various cellular processes, including transcription, intracellular trafficking, and chromosome remodeling [3, 12]. In addition, lncRNAs functioning as regulatory factors have been determined for several complex cellular processes, such as cell death, growth, differentiation, identity establishment; controlling apoptosis, epigenetic regulation, genomic imprinting, alternative splicing, regulation of gene expression at posttranscriptional level, chromatin modification, inflammatory pathologies, and, when deregulated, also in various cancer types [13–23].

lncRNAs can be present in practically all cell compartments [24]. However, many lncRNAs with high abundance were identified especially in the nucleus and cytoplasm [25, 26]. lncRNA secondary structures, such as stem loops and hairpins, results of posttranscriptional modifications, enable their interaction with proteins and chromatin and are crucial for lncRNA's vast set of functions [12]. Some of the main mechanisms of action that allow lncRNAs to have a crucial role in various cellular processes [27] are presented in Figure 1. In general, lncRNAs may act as scaffolds for grouping protein complexes (Figure 1(b)), guides to recruit proteins (Figure 1(c)), transcriptional enhancers by bending chromatin (Figure 1(d)), decoys to release proteins from chromatin (Figure 1(a)), or antagonists for other regulatory ncRNAs, for example, microRNAs (miRNAs) [12, 28].

Regardless of the whole human genome analyses that enabled better understanding of lncRNA expression, function, and distribution in the human genome, classification of lncRNAs remains to be unified [25, 29]. lncRNAs can be

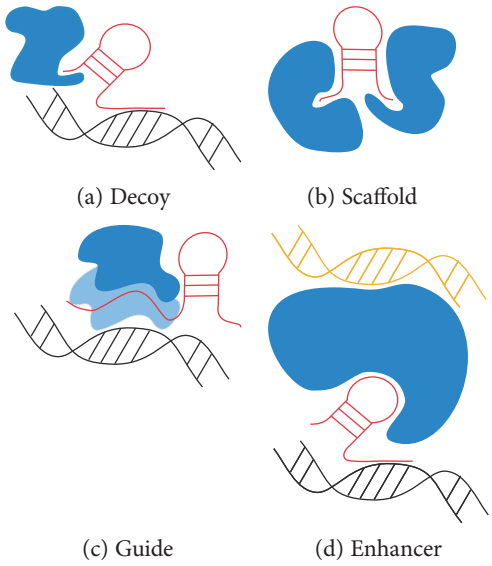

Figure 1: Different mechanisms of long noncoding RNA (lncRNA) action. (a) The lncRNAs can act as decoys, titrating away DNA-binding proteins (e.g., transcription factors); (b) lncRNAs may act as scaffolds to bring two or more proteins to spatial proximity or into a complex; (c) lncRNAs may act as guides to recruit proteins to DNA (e.g., chromatin modification enzymes); (d) lncRNA guidance can also be exerted through chromosome looping in an enhancer-like model in *cis*. lncRNA (red); DNA (black); section of DNA loop (yellow); DNA-binding proteins (blue and light blue). The figure is adapted from John L. Rinn and Howard Y. Chang [12].

sorted according to their structure, sequence, function, localization, metabolism, and interaction with protein-coding genes or other DNA elements [29]. Recently, Wang et al. classified different types of lncRNAs according to their genomic location and context, exerted effect on DNA sequences, mechanism of functioning, and targeting mechanism [30]. In addition, lncRNAs can be classified into several categories including sense lncRNAs, antisense lncRNAs, bidirectional lncRNAs, intronic lncRNAs, intergenic lncRNAs, promoter-associated lncRNAs, and untranslated region-(UTR-) associated lncRNAs [25, 26]. Nevertheless, current classification methods remain inadequate and relatively nontransparent. The general long-term goal is to develop a unified, systematic, and comprehensive lncRNA classification and annotation framework, utilizing global system biology and genomics-driven approaches. Also, the development of improved tools is required for the integration of complex data from multiple types of experiments into this framework, revealing associations between coding and noncoding transcripts. Such lncRNA classification would be a prerequisite for an improved overview and more effective access and usage of large-scale lncRNA data in various fields and applications [29].

Association of lncRNAs in carcinogenesis was observed due to their differential expression in tumors when compared to normal tissues [31]. lncRNAs H19, MALAT1, and PCA3 are highly expressed tumor-associated lncRNAs that were characterized before the availability of next generation sequencing technologies [32–34]. It has been demonstrated that tumorigenesis mostly results from ectopic lncRNA expression [35]. lncRNAs regulate several oncogenes and tumor suppressor genes at transcriptional and posttranscriptional levels, affecting proliferation, apoptosis, angiogenesis, invasion, migration, and metastasis of tumor cells [36–39]. Also, lncRNA-mediated regulation of chromatin remodeling is essential for the integrity of nuclear structure [23]. In recent years, next-generation and high-throughput sequencing techniques have enabled a significant breakthrough in lncRNA identification and characterization. This resulted in continuously rising amounts of data elucidating deregulated lncRNAs associated with the development of various cancer types [40–42]. In this review, we primarily focus on describing circulating lncRNAs present in different body fluids which represent a promising category of biomarkers for cancer diagnosis, prognosis, and also treatment.

2. lncRNA-Mediated Epigenetic Modifications

Cancer development and progression can be mediated through multiple mechanisms involving lncRNAs [36, 43–46]. In particular, involvement of lncRNAs has been extensively studied in cancer progression, mainly through epigenetic regulation, activation of oncogenic pathways, and crosstalk with other RNA subtypes [29, 47, 48]. As mentioned before, lncRNAs can interact with chromatin remodeling complexes which usually leads to modifications in the expression of target genes, in either *cis* or *trans* [49]. In these processes, lncRNAs usually recruit chromatin modification factors, for example, DNA methyltransferase enzymes [50], resulting in gene expression variations often inherited within cell lineages [51]. One of the first reported and characterized lncRNA involved in cancer progression through genome-wide epigenetic reprogramming was HOTAIR [52–54]. HOTAIR acts through interaction with polycomb repressive complex 2 (PRC2) subunits, a key chromatin remodeling complex involved in gene silencing [55]. When deregulated, HOTAIR recruits PRC2 subunits in promoter regions of tumor suppressor genes which results in their transcriptional repression and chromatin condensation, thus, favoring tumor progression. Studies have shown that beside HOTAIR, ANRIL, and XIST, lncRNAs also recruit PRC2 in a similar fashion [52, 56, 57].

Studies have shown that over 200 lncRNAs participate in imprinting processes where, depending on their parental origins, specific expression of nearby lncRNAs promotes suppression of neighboring genes in *cis* [58, 59]. Here, instead of acting through, for example, PRC2, lncRNAs recruit DNA methyltransferases directly to modify chromatin conformation and DNA methylation. Among many lncRNAs with such function, several have been characterized, including Kcnq1ot1, TARID, H19, AS1DHRS4, and DACOR1. lncRNAs may also modify nucleosome positioning through SWI/SNF complexes as it was determined for SChLAP1 [60–65]. lncRNA SChLAP1 is overexpressed in a subset of prostate cancers. SChLAP1 can bind directly to hSNF5, one of the core subunits of the SWI/SNF complexes, thus, decreasing their genomic binding. By impairing the proper SWI/SNF regulation of gene expression, SChLAP1

antagonizes tumor suppressive function of the SWI/SNF complexes and promotes tumor cell invasion and metastasis [63, 66]. In addition, NEAT1, UCA1, HIF1A-AS1, and Evf2 also interact with core subunits of SWI/SNF complexes in a similar manner in various cancer types [67]. Other lncRNAs, including Firre, bind chromatin remodelers cohesin and CTCF in order to change the chromatin of whole chromosomes in the process of X chromosome inactivation [68]. lncRNAs may also act as chromatin activators, regulating chromosome looping in their proximity to deposit activating H3K4me3 histone mark on gene promoters [69–71].

3. Circulating lncRNAs as Biomarkers in Cancer

Among the main advantages of lncRNAs that make them suitable as cancer diagnostic and prognostic biomarkers is their high stability while circulating in body fluids, especially when included in exosomes or apoptotic bodies [72]. Studies have shown that despite abundant quantities of ribonucleases in different body fluids, lncRNAs were detected in these samples which could successfully resist ribonuclease degradation activities [35]. In addition, lncRNA deregulation in primary tumor tissues is clearly mirrored in various bodily fluids, including whole blood, plasma, urine, saliva, and gastric juice [73–76]. These lncRNA characteristics present an opportunity to develop effective and convenient lncRNA-based biomarkers that are minimally invasive and may be better tolerated by patients, when compared to conventional biopsies, due to their relative noninvasiveness [77]. Detection of circulating cancer-associated lncRNAs in body fluids could be used in the assessment of cancers at distinguishing tumor patients from healthy people at early stages with both high sensitivity and specificity. In addition, predicting the prognosis of tumor patients and the risk of tumor metastasis and recurrence after surgery could be assessed, along with evaluating operation success [35]. Several individual or combined lncRNAs have demonstrated comparable or, in some cases, even higher diagnostic performance than conventional cancer biomarkers, for different cancer types. lncRNA MALAT1 has been identified, by Kaplan-Meier analysis, as an effective prognostic parameter for patient survival in stage I nonsmall cell lung cancer [78]. Also, the measurement of lncRNA PCA3 in patient urine samples has been shown to allow more sensitive and specific diagnosis of prostate cancer than the widely used prostate-specific antigen (PSA) serum levels [79–81]. CEA, CA125, CA153, and AFP are conventional biomarkers, commonly used for breast cancer diagnosis. lncRNA RP11-445H22.4 is overexpressed in breast cancer tissues and can be detected in serum samples, with a sensitivity of 92% and specificity of 74%, which is significantly better than the performance of above listed conventional biomarkers [82]. In addition, diagnostic performances of lncRNAs TINCR, CCAT2, AOC4P, BANCR, LINC00857, AA174084, and H19 were evaluated in body fluid samples (e.g., plasma and gastric juice) of gastric cancer patients. These lncRNAs had the ability to differentiate gastric cancer patients from healthy individuals and to effectively detect different stages of gastric cancer (from early to metastatic cancer forms). However, despite their overall positive diagnostic performances, similar to those obtained by several conventional cancer biomarkers, false-positive and false-negative detections were observed [19, 76, 83]. Also, similar results were obtained after characterizing lncRNAs MALAT1 and PCA3 as biomarkers in prostate cancer patients [84, 85].

Stability of lncRNAs in body fluids of tumor patients has not been thoroughly explored. Studies revealed that some lncRNAs remained stable in plasma under extreme conditions, including several freeze-thawed cycles and prolonged incubation at elevated temperatures [86]. It has also been demonstrated that lncRNAs remained their stability when using plasma and serum from EDTA vacutainer tubes or from tubes lacking the specific anticoagulant, whereas lncRNA amounts declined when using plasma from heparin vacutainer tubes [84].

Three main mechanisms for lncRNA secretion and transport to the extracellular environment have been proposed. First, extracellular RNAs may package themselves into specific membrane vesicles, such as exosomes and microvesicles, in order to be secreted and resist RNase activity. Studies revealed that exosomes most frequently protect plasma lncRNAs [87–90]. Second, extracellular RNAs can be actively released by tumor tissues and cells [84]. However, elevated values of lncRNAs in plasma may have multiple sources, including cancer-adjacent normal cells, immune cells, and other blood cells [86, 90]. Third, extracellular RNAs may encapsulate themselves into high-density lipoprotein (HDL) or apoptotic bodies or are associated with protein complexes, for example, Argonaute- (Ago-) miRNA complex [91] and nucleophosmin 1- (NPM1-) miRNA complex [92]. However, despite many performed studies, secretion and transport mechanisms of lncRNAs to the circulation system remain poorly understood, mostly because several studies tend to contradict each other. Also, thorough examinations and reports regarding biological functions of lncRNAs in cancers are still lacking [35].

In order to introduce circulating lncRNAs into clinical practice, further studies and improvements should be performed regarding the standardization of sample preparation protocols, endogenous controls of lncRNAs in body fluids and the extraction methods should be uniformed, standards assessing the quality of lncRNAs and the credibility of qPCR results should be more accurate and reliable, and more high-quality research studies should be performed, with selection bias reduced as much as possible [35]. In addition, several technical obstacles remain to be addressed and overcome in the future, to enable a reliable use of circulating lncRNAs as effective cancer biomarkers. Commercial kits employing columns are mostly used for lncRNA extraction from body fluids. Unfortunately, no consistent results have been obtained regarding the differences in the efficiency of column-based methods, indicating that comparison and standardization of lncRNA extraction methods are necessary [93]. Absolute concentration of lncRNAs in body fluids is usually low and frequently requires an RNA amplification step prior analysis, which is time consuming and can be problematic when results are needed promptly [94]. It has also been observed that RNA extracted from plasma and serum samples may be undetectable when using a NanoDrop

Table 1: Upregulated cancer-associated lncRNAs when compared to normal tissues.

Name	Cancer type	Fold change[a]	References
Wt1-as	Acute myeloid leukemia	NA[b]	[111]
XIST	Glioma	NA	[112]
CRNDE	Glioma	NA	[113]
MALAT1	Glioma	2.0–5.0	[114]
	Colorectal	2.0–6.0	[115]
	Lung	>40.0	[116]
	Prostate	NA	[84]
	Hepatocellular	NA	[117]
	Uterus	NA	[118]
LSINCT5	Breast	2.0–7.0	[119]
LINC00617	Breast	>1.5	[120]
RP11-445H22.4	Breast	15.0–20.0	[121]
BC200	Breast	NA	[122]
CCHE1	Cervical	NA	[123]
CCAT1-L	Colorectal	NA	[71]
POU3F3	Esophageal	NA	[124]
PCAT-1	Colorectal	NA	[125]
	Prostate	NA	[126]
HOTAIR	Esophageal	NA	[127]
	Lung	NA	[128]
	Cervical	NA	[129]
	Pancreas	NA	[130]
	Breast	NA	[52]
	Oral	NA	[131]
	Hepatocellular	>2.0	[132]
	Glioma	NA	[133, 134]
	Colorectal	5.2	[101]
CCAT2	Lung	7.5	[135]
	Colon	NA	[136]
	Cervical	NA	[137]
LINC00152	Gastric	NA	[35, 90]
LSINCT-5	Gastric	NA	[138]
HOXA11-AS	Glioma	NA	[139]
Linc-POU3F3	Glioma	>2.6	[140]
ATB	Glioma	5.0–10.0	[141]
AB073614	Glioma	NA	[142]
RP11-160H22.5	Hepatocellular	2.5	[109]
XLOC_014172	Hepatocellular	67.7	[109]
LOC149086	Hepatocellular	4.6	[109]
BANCR	Hepatocellular	NA	[143]
SNHG3	Hepatocellular	NA	[144]
MVIH	Hepatocellular	3.75	[145]
	Lung	NA	[146]
LCAL1	Lung	NA	[147]
LUADT1	Lung	NA	[148]
AFAP1-AS1	Lung	NA	[149]
	Colorectal	NA	[150]
	Hepatocellular	NA	[151]
	Esophageal	>1.0	[152]
ANRIL	Lung	>1.5	[153]
	Hepatocellular	>1.0	[154]
	Bladder	>1.0	[155]
UCA1	Lung	NA	[156]
	Oral	NA	[157]
	Bladder	32.9	[158]
	Colon	NA	[159]
	Hepatocellular	NA	[160]
	Breast	NA	[161]
	Esophageal	>2.0	[162]
CASC15	Melanoma	NA	[163]
SPRY4-IT1	Melanoma	>2.0	[164, 165]
	Glioma	NA	[166]
H19	Bladder	NA	[167]
	Gastric	NA	[83]
	Esophageal	NA	[168]
	Colorectal	NA	[169]
	Glioma	NA	[170]
HULC	Pancreas	NA	[171]
	Hepatocellular	32.7	[102]
	Glioma	NA	[172]
PCA3	Prostate	NA	[34, 99]
PCAT5	Prostate	NA	[173]
PCAT18	Prostate	8.8–11.1	[174]
PRNCR1	Prostate	NA	[175]
NEAT1	Glioma	NA	[176, 177]
	Oral	NA	[74]
	Hepatocellular	NA	[178]
	Nasopharyngeal	NA	[179]
PVT1	Thyroid	NA	[180]
	Gastric	NA	[181]
	Colorectal	NA	[182]
SRA	Breast	NA	[183]

[a]Fold change values, relative to normal controls; [b]not available (data is presented in a graphical format in the original report).

spectrophotometer for quantifying circulating RNAs [93]. This makes the necessity for the development of highly sensitive methods for quantifying lncRNAs crucial. Also, since the mechanisms of lncRNA secretion are not yet fully understood, the levels of circulating lncRNAs may be affected by other concomitant disease changes, besides tumorigenesis. Thus, overrated amounts of specific lncRNAs associated with a particular disease may be determined [94]. There are also several existing obstacles regarding the techniques, commonly used for quantifying circulating lncRNAs. Quantitative RT-PCR is a well-established method for detecting and

quantifying circulating RNAs. However, the cost per sample is relatively high and the throughput of the method low [93]. Recently developed assays, such as the Human Disease-Related lncRNA Profiler (System Biosciences SBI), allow the measurement of a panel of lncRNAs but can detect only annotated lncRNAs. Therefore, only a medium throughput can be attained [93]. Commercial lncRNA microarray platforms can be used to detect only previously described biomarkers already present in the lncRNA databases. Microarrays have a high throughput, but a lower dynamic range and specificity, when compared to qRT-PCR and RNA-seq [93]. RNA-seq can be used for the identification of known and new lncRNA species, with lower cost per sample than microarrays and qRT-PCR. However, a relatively large amount of starting material is required (cca. 1 μg RNA), which is difficult to extract from biological fluids, for example, plasma or serum samples. In addition, current RNA-seq methodology is expensive and complex and requires a special equipment with a trained personnel [93].

Since expression profiles of cancer-associated lncRNAs may be very specific for various cancer types, these specific lncRNAs could be efficiently used as tumor biomarkers in different body fluids in the near future, with vital significance for clinical research [35]. In the following section, we describe some of these lncRNAs.

Table 2: Downregulated cancer-associated lncRNAs when compared to normal tissues.

Name	Cancer type	Fold change[a]	References
MEG3	Glioma	NA[b]	[184, 185]
ZFAS1	Breast	2.0	[186]
GAS5	Breast	<1.0	[187]
	Glioma	NA	[188]
LOC554202	Colorectal	NA	[189]
CUDR	Gastric	NA	[190]
PTENP1	Gastric	NA	[191]
	Prostate	NA	[191]
AA174084	Gastric	3.2	[76]
LINC00982	Gastric	7.7	[192]
TSLC1-AS1	Glioma	NA	[193]
ADAMTS9-AS2	Glioma	NA	[194]
MDC1-AS	Glioma	NA	[195]
TUG1	Glioma	NA	[196]
ROR	Glioma	NA	[197]
CACS2	Glioma	NA	[198]
PRAL	Hepatocellular	NA	[199]
MALAT1	Lung	3.3	[106]
AK023948	Papillary thyroid carcinoma	5.0	[200]

[a]Fold change values, relative to normal controls; [b]not available (data is presented in a graphical format in the original report).

4. lncRNAs as Cancer Biomarkers Obtained from Body Fluids

Deregulated expression of lncRNAs is strongly linked to the development of various tumors and can be relatively effectively detected in patient's body fluids for several cancer types [77]. Regarding their involvement in malignant disease development, when comparing to normal tissues of healthy individuals, lncRNAs are generally divided into oncogenic or tumor suppressive, being upregulated or downregulated, respectively [31, 45]. Sets of a number of differentially expressed cancer-associated lncRNAs in a variety of cancers are presented in Tables 1 and 2. Among them, several lncRNAs represent promising noninvasive cancer biomarkers for detection in patient's body fluids, including PCA3, HOTAIR, HULC, MALAT1, H19, LINC00152, RP11-160H22.5, XLOC_014172, LOC149086, AA174084, and UCA1. Moreover, for several of these lncRNAs, it has been already demonstrated that they could be effectively used as diagnostic and prognostic cancer biomarkers in clinical practice.

PCA3 has been recently approved as a urine biomarker for prostate cancer by the US Food and Drug Administration [73]. This lncRNA allows better sensitivity and specificity when compared to the widely used PSA blood test, mainly because of its significantly higher expression in prostate cancer patients [79–81, 95–97]. A meta-analysis of several studies has determined the validity of PCA3 levels in urine samples for prostate cancer diagnosis, with a summary sensitivity of 62% and specificity of 75%. In the receiver operating characteristic (ROC) curve analysis, this translated to an area under the ROC curve (AUC) of 0.75 [98]. PCA3 has also a prognostic value for prostate cancer, since its expression levels correlate well with tumor aggressiveness [99, 100].

HOTAIR was found to be highly expressed in saliva samples of oral squamous cell carcinoma (OSCC) patients. Since higher expression levels of HOTAIR were determined for metastatic patients, this lncRNA represents a strong candidate for metastatic oral cancer diagnosis [74]. In addition, the association between increased blood levels of HOTAIR and poor prognosis with higher mortality in colorectal cancer patients has been determined. Expression levels of HOTAIR could also predict the survival time of patients. Evaluated diagnostic performance of HOTAIR in peripheral blood cells has shown its sensitivity of 67%, specificity of 92.5%, and AUC of 0.87. Thus, HOTAIR represents an effective negative prognostic biomarker for colorectal cancer in blood samples [101].

HULC can be effectively detected in plasma and peripheral blood cells and is significantly overexpressed in hepatocellular carcinoma patients, thus, representing a prominent novel biomarker for liver cancer. However, no data regarding HULC diagnostic performance are available at this time [102, 103]. HULC detected in blood has also been recently proposed as a diagnostic biomarker for gastric cancer [104].

MALAT1 represents a promising diagnostic biomarker detectable in blood, to effectively screen lung cancer. One study has shown downregulation of MALAT1 in blood samples of lung cancer patients which was contrary to MALAT1 levels in lung cancer tissues, where it was significantly

Table 3: Databases containing lncRNA data.

Name	URL	References
CHIPbase	http://deepbase.sysu.edu.cn/chipbase/	[201]
C-It-Loci	http://c-it-loci.uni-frankfurt.de/	[202]
Co-LncRNA	http://www.bio-bigdata.com/Co-LncRNA/	[203]
DIANA-LncBase	http://www.microrna.gr/LncBase	[204, 205]
Linc2GO	http://www.bioinfo.tsinghua.edu.cn/~liuke/Linc2GO/index.html	[206]
Lnc2Cancer	http://www.bio-bigdata.com/lnc2cancer/	[207]
LncACTdb	http://www.bio-bigdata.net/LncACTdb/	[208]
LNCipedia	http://www.lncipedia.org/	[209, 210]
LncRBase	http://bicresources.jcbose.ac.in/zhumur/lncrbase/	[211]
LncRNA2Function	http://mlg.hit.edu.cn/lncrna2function/	[212]
LncRNAdb	http://www.lncrnadb.org/	[213, 214]
LncRNADisease	http://210.73.221.6/lncrnadisease	[215]
lncRNASNP	http://bioinfo.life.hust.edu.cn/lncRNASNP/	[216]
LncRNome	http://genome.igib.res.in/lncRNome/	[217]
miRcode	http://www.mircode.org	[218]
NONCODE	http://www.noncode.org/	[219, 220]
Starbase 2.0v	http://starbase.sysu.edu.cn/rbpLncRNA.php	[221, 222]

upregulated. Conversely, MALAT1 showed elevated expression levels in whole blood of metastatic lung cancer patients [105]. Due to its relatively low expression and low detection sensitivity (sensitivity 56%; specificity 96%; AUC 0.79) in diagnosis of non-small-cell lung cancer (NSCLC), MALAT1 is not regarded suitable as an independent biomarker to diagnose lung cancer but should be rather used as a complementary biomarker [106]. In addition to lung cancer, MALAT1 has proven itself as a prominent biomarker with its elevated expression detected in plasma and urine of prostate cancer patients, with a sensitivity and specificity of 58.6% and 84.8%, respectively (AUC 0.836). MALAT1 also helped to predict the outcome of prostate biopsies [84, 107].

Elevated expression profiles of H19 have been determined in plasma samples of gastric cancer patients. H19 has great potential as a promising biomarker due to its high diagnostic value for the detection of gastric cancer (sensitivity 82.9%; specificity 72.9%; AUC 0.838). It has also been more effective in early stage gastric cancer diagnosis than the conventional biomarkers, such as CEA and CA199, with a sensitivity of 85.5%, specificity of 80.1%, and AUC of 0.877 [83].

Expression levels of LINC00152 in plasma were found to be significantly increased in early and advanced gastric cancer patients. This lncRNA had also significantly higher expression profiles in postoperative plasma samples. The diagnostic value of LINC00152 (sensitivity 48.1%; specificity 85.2%; AUC 0.675) was better than those of CEA and CA199 biomarkers, which makes LINC00152 a good candidate as a novel blood-based biomarker for gastric cancer diagnosis [90]. In addition, LINC00152 could also be detected in the gastric juice of patients with gastric cancer [108].

Among the less commonly studied lncRNAs belong RP11-160H22.5, XLOC_014172, and LOC149086 which have been proposed as biomarkers for the diagnosis of hepatocellular carcinoma in patient plasma samples. These three lncRNAs had better scores for hepatocellular carcinoma diagnosis when used in combination, in comparison to each individual lncRNA, with a merged AUC of 0.896, sensitivity of 82%, and specificity of 73% [109]. In addition, XLOC_014172 and LOC149086 lncRNAs had also a good prognostic value for metastasis prediction (sensitivity 91%; specificity 90%; AUC for the combined lncRNAs 0.675) [109].

AA174084 represents a relatively robust but specific biomarker, suitable for the diagnosis of gastric cancer in gastric juice samples (sensitivity 46%; specificity 93%; AUC 0.848). Levels of AA174084 in patient's gastric juices were found to be significantly upregulated when compared to those of healthy individuals. Interestingly, this lncRNA was not suitable for the diagnosis of gastric cancer from plasma samples [76].

UCA1 lncRNA has been identified as a potential biomarker for bladder cancer. Due to its relatively high overall specificity, it has a high potential to discriminate between the bladder/urothelial cancer and other cancer types, or other diseases related to the urinary tract (sensitivity 80.9%; specificity 91.8%; AUC 0.882). UCA1 can be detected in urine samples of bladder cancer patients, mostly in the cellular sediments [110].

Additional, continuously increasing amounts of information regarding cancer-associated lncRNAs, including those detected in body fluids, can be obtained from many existing databases, several of which are presented in Table 3.

5. Conclusions and Perspectives

lncRNAs represent a relatively large and heterogeneous group of ncRNAs and are considered as suitable diagnostic

and prognostic biomarkers in cancer. In recent years, circulating lncRNAs have proven themselves extremely valuable for the detection of various cancer types. Their usage as biomarkers is convenient not only because samples containing circulating lncRNAs can be easily and noninvasively obtained from cancer patients but also because these lncRNAs remain relatively stable in body fluids. They can be quite easily detected in whole blood, plasma, serum, urine, saliva, and gastric juice samples, by using a variety of common molecular biology techniques, such as qRT-PCR, microarray hybridization, and sequencing (e.g., RNA-seq). Because lncRNAs are usually differentially abundant in different body fluids, mainly depending on the cancer type, effective cancer diagnosis and prognosis currently depend on combining different candidate lncRNAs, together with previously established biomarkers. Some circulating lncRNAs have already been proven as promising and sensitive biomarkers, and there are likely more to come.

Conflicts of Interest

The authors declare that they have no competing interests.

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References

[1] J. S. Mattick, "Non-coding RNAs: the architects of eukaryotic complexity," *EMBO Reports*, vol. 2, no. 11, pp. 986–991, 2001.

[2] C. Lee and N. Kikyo, "Strategies to identify long noncoding RNAs involved in gene regulation," *Cell & Bioscience*, vol. 2, no. 1, p. 37, 2012.

[3] J. J. Quinn and H. Y. Chang, "Unique features of long noncoding RNA biogenesis and function," *Nature Reviews. Genetics*, vol. 17, no. 1, pp. 47–62, 2016.

[4] J. Harrow, A. Frankish, J. M. Gonzalez et al., "GENCODE: the reference human genome annotation for the ENCODE project," *Genome Research*, vol. 22, no. 9, pp. 1760–1774, 2012.

[5] T. Alam, Y. A. Medvedeva, H. Jia, J. B. Brown, L. Lipovich, and V. B. Bajic, "Promoter analysis reveals globally differential regulation of human long non-coding RNA and protein-coding genes," *PloS One*, vol. 9, no. 10, article e109443, 2014.

[6] O. Wapinski and H. Y. Chang, "Long noncoding RNAs and human disease," *Trends in Cell Biology*, vol. 21, no. 6, pp. 354–361, 2011.

[7] C. A. Brosnan and O. Voinnet, "The long and the short of noncoding RNAs," *Current Opinion in Cell Biology*, vol. 21, no. 3, pp. 416–425, 2009.

[8] C. I. Brannan, E. C. Dees, R. S. Ingram, and S. M. Tilghman, "The product of the H19 gene may function as an RNA," *Molecular and Cellular Biology*, vol. 10, no. 1, pp. 28–36, 1990.

[9] N. Brockdorff, A. Ashworth, G. F. Kay et al., "The product of the mouse Xist gene is a 15 kb inactive X-specific transcript containing no conserved ORF and located in the nucleus," *Cell*, vol. 71, no. 3, pp. 515–526, 1992.

[10] H. van Bakel and T. R. Hughes, "Establishing legitimacy and function in the new transcriptome," *Briefings in Functional Genomics & Proteomics*, vol. 8, no. 6, pp. 424–436, 2009.

[11] Y. Okazaki, M. Furuno, T. Kasukawa et al., "Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs," *Nature*, vol. 420, no. 6915, pp. 563–573, 2002.

[12] J. L. Rinn and H. Y. Chang, "Genome regulation by long noncoding RNAs," *Annual Review of Biochemistry*, vol. 81, pp. 145–166, 2012.

[13] W. Hu, J. R. Alvarez-Dominguez, and H. F. Lodish, "Regulation of mammalian cell differentiation by long non-coding RNAs," *EMBO Reports*, vol. 13, no. 11, pp. 971–983, 2012.

[14] R. A. Flynn and H. Y. Chang, "Long noncoding RNAs in cell-fate programming and reprogramming," *Cell Stem Cell*, vol. 14, no. 6, pp. 752–761, 2014.

[15] M. N. Rossi and F. Antonangeli, "LncRNAs: new players in apoptosis control," *International Journal of Cell Biology*, vol. 2014, Article ID 473857, 7 pages, 2014.

[16] L. W. Harries, "Long non-coding RNAs and human disease," *Biochemical Society Transactions*, vol. 40, no. 4, pp. 902–906, 2012.

[17] K. Qian, G. Liu, Z. Tang et al., "The long non-coding RNA NEAT1 interacted with miR-101 modulates breast cancer growth by targeting EZH2," *Archives of Biochemistry and Biophysics*, vol. 615, pp. 1–9, 2017.

[18] B. Liu, C. F. Pan, Z. C. He et al., "Long noncoding RNA-LET suppresses tumor growth and EMT in lung adenocarcinoma," *BioMed Research International*, vol. 2016, Article ID 4693471, 9 pages, 2016.

[19] K. Zhang, H. Shi, H. Xi et al., "Genome-wide lncRNA microarray profiling identifies novel circulating lncRNAs for detection of gastric cancer," *Theranostics*, vol. 7, no. 1, pp. 213–227, 2017.

[20] Z. Y. Yang, F. Yang, Y. L. Zhang et al., "LncRNA-ANCR down-regulation suppresses invasion and migration of colorectal cancer cells by regulating EZH2 expression," *Cancer Biomarkers : Section A of Disease Markers*, vol. 18, no. 1, pp. 95–104, 2017.

[21] P. Kapranov, J. Cheng, S. Dike et al., "RNA maps reveal new RNA classes and a possible function for pervasive transcription," *Science (New York, N.Y.)*, vol. 316, no. 5830, pp. 1484–1488, 2007.

[22] R. Spizzo, M. I. Almeida, A. Colombatti, and G. A. Calin, "Long non-coding RNAs and cancer: a new frontier of translational research?" *Oncogene*, vol. 31, no. 43, pp. 4577–4587, 2012.

[23] J. E. Wilusz, H. Sunwoo, and D. L. Spector, "Long noncoding RNAs: functional surprises from the RNA world," *Genes & Development*, vol. 23, no. 13, pp. 1494–1504, 2009.

[24] R. Flippot, G. G. Malouf, X. Su, R. Mouawad, J. P. Spano, and D. Khayat, "Cancer subtypes classification using long non-coding RNA," *Oncotarget*, vol. 7, no. 33, pp. 54082–54093, 2016.

[25] T. Derrien, R. Johnson, G. Bussotti et al., "The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression," *Genome Research*, vol. 22, no. 9, pp. 1775–1789, 2012.

[26] C. P. Ponting, P. L. Oliver, and W. Reik, "Evolution and functions of long noncoding RNAs," *Cell*, vol. 136, no. 4, pp. 629–641, 2009.

[27] M. Guttman and J. L. Rinn, "Modular regulatory principles of large non-coding RNAs," *Nature*, vol. 482, no. 7385, pp. 339–346, 2012.

[28] T. R. Mercer, M. E. Dinger, and J. S. Mattick, "Long noncoding RNAs: insights into functions," *Nature Reviews. Genetics*, vol. 10, no. 3, pp. 155–159, 2009.

[29] G. St Laurent, C. Wahlestedt, and P. Kapranov, "The landscape of long non-coding RNA classification," *Trends in Genetics : TIG*, vol. 31, no. 5, pp. 239–251, 2015.

[30] Q. Wang, S. Gao, H. Li, M. Lv, and C. Lu, "Long noncoding RNAs (lncRNAs) in triple negative breast cancer," *Journal of Cellular Physiology*, vol. 9999, pp. 1–8, 2017.

[31] N. Bartonicek, J. L. V. Maag, and M. E. Dinger, "Long noncoding RNAs in cancer: mechanisms of action and technological advancements," *Molecular Cancer*, vol. 15, no. 1, p. 43, 2016.

[32] Y. Zhang, T. Shields, T. Crenshaw, Y. Hao, T. Moulton, and B. Tycko, "Imprinting of human H19: allele-specific CpG methylation, loss of the active allele in Wilms tumor, and potential for somatic allele switching," *American Journal of Human Genetics*, vol. 53, no. 1, pp. 113–124, 1993.

[33] J. H. Luo, B. Ren, S. Keryanov et al., "Transcriptomic and genomic analysis of human hepatocellular carcinomas and hepatoblastomas," *Hepatology (Baltimore, Md.)*, vol. 44, no. 4, pp. 1012–1024, 2006.

[34] J. B. de Kok, G. W. Verhaegh, R. W. Roelofs et al., "DD3(PCA3), a very sensitive and specific marker to detect prostate tumors," *Cancer Research*, vol. 62, no. 9, pp. 2695–2698, 2002.

[35] T. Shi, G. Gao, and Y. Cao, "Long noncoding RNAs as novel biomarkers have a promising future in cancer diagnostics," *Disease Markers*, vol. 2016, Article ID 9085195, 10 pages, 2016.

[36] H. Zhang, Z. Chen, X. Wang, Z. Huang, Z. He, and Y. Chen, "Long non-coding RNA: a new player in cancer," *Journal of Hematology & Oncology*, vol. 6, no. 1, p. 37, 2013.

[37] M. T. Qiu, J. W. Hu, R. Yin, and L. Xu, "Long noncoding RNA: an emerging paradigm of cancer research," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 34, no. 2, pp. 613–620, 2013.

[38] S. Xu, S. Sui, J. Zhang et al., "Downregulation of long noncoding RNA MALAT1 induces epithelial-to-mesenchymal transition via the PI3K-AKT pathway in breast cancer," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 5, pp. 4881–4891, 2015.

[39] Q. Ji, X. Liu, X. Fu et al., "Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/beta-catenin signal pathway," *PloS One*, vol. 8, no. 11, article e78700, 2013.

[40] M. K. Iyer, Y. S. Niknafs, R. Malik et al., "The landscape of long noncoding RNAs in the human transcriptome," *Nature Genetics*, vol. 47, no. 3, pp. 199–208, 2015.

[41] X. Yan, Z. Hu, Y. Feng et al., "Comprehensive genomic characterization of long non-coding RNAs across human cancers," *Cancer Cell*, vol. 28, no. 4, pp. 529–540, 2015.

[42] M. B. Clark, T. R. Mercer, G. Bussotti et al., "Quantitative gene profiling of long noncoding RNAs with targeted RNA sequencing," *Nature Methods*, vol. 12, no. 4, pp. 339–342, 2015.

[43] S. W. Cheetham, F. Gruhl, J. S. Mattick, and M. E. Dinger, "Long noncoding RNAs and the genetics of cancer," *British Journal of Cancer*, vol. 108, no. 12, pp. 2419–2425, 2013.

[44] E. A. Gibb, C. J. Brown, and W. L. Lam, "The functional role of long non-coding RNA in human carcinomas," *Molecular Cancer*, vol. 10, no. 1, p. 38, 2011.

[45] N. Hauptman and D. Glavac, "Long non-coding RNA in cancer," *International Journal of Molecular Sciences*, vol. 14, no. 3, pp. 4655–4669, 2013.

[46] S. A. Mitra, A. P. Mitra, and T. J. Triche, "A central role for long non-coding RNA in cancer," *Frontiers in Genetics*, vol. 3, no. 17, p. 70, 2012.

[47] G. A. Calin, C. G. Liu, M. Ferracin et al., "Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas," *Cancer Cell*, vol. 12, no. 3, pp. 215–229, 2007.

[48] Y. F. Gao, Z. B. Wang, T. Zhu et al., "A critical overview of long non-coding RNA in glioma etiology 2016: an update," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 11, pp. 14403–14413, 2016.

[49] A. Fatica and I. Bozzoni, "Long non-coding RNAs: new players in cell differentiation and development," *Nature Reviews. Genetics*, vol. 15, no. 1, pp. 7–21, 2014.

[50] F. Lai and R. Shiekhattar, "Where long noncoding RNAs meet DNA methylation," *Cell Research*, vol. 24, no. 3, pp. 263-264, 2014.

[51] C. Martin and Y. Zhang, "Mechanisms of epigenetic inheritance," *Current Opinion in Cell Biology*, vol. 19, no. 3, pp. 266–272, 2007.

[52] R. A. Gupta, N. Shah, K. C. Wang et al., "Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis," *Nature*, vol. 464, no. 7291, pp. 1071–1076, 2010.

[53] L. Lu, G. Zhu, C. Zhang et al., "Association of large noncoding RNA HOTAIR expression and its downstream intergenic CpG island methylation with survival in breast cancer," *Breast Cancer Research and Treatment*, vol. 136, no. 3, pp. 875–883, 2012.

[54] K. P. Sorensen, M. Thomassen, Q. Tan et al., "Long non-coding RNA HOTAIR is an independent prognostic marker of metastasis in estrogen receptor-positive primary breast cancer," *Breast Cancer Research and Treatment*, vol. 142, no. 3, pp. 529–536, 2013.

[55] F. C. Beckedorff, M. S. Amaral, C. Deocesano-Pereira, and S. Verjovski-Almeida, "Long non-coding RNAs and their implications in cancer epigenetics," *Bioscience Reports*, vol. 33, no. 4, article e00061, 2013.

[56] J. L. Rinn, M. Kertesz, J. K. Wang et al., "Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs," *Cell*, vol. 129, no. 7, pp. 1311–1323, 2007.

[57] C. Kanduri, J. Whitehead, and F. Mohammad, "The long and the short of it: RNA-directed chromatin asymmetry in mammalian X-chromosome inactivation," *FEBS Letters*, vol. 583, no. 5, pp. 857–864, 2009.

[58] J. Cao, "The functional role of long non-coding RNAs and epigenetics," *Biological Procedures Online*, vol. 16, no. 1, p. 11, 2014.

[59] Y. He, X. M. Meng, C. Huang et al., "Long noncoding RNAs: novel insights into hepatocelluar carcinoma," *Cancer Letters*, vol. 344, no. 1, pp. 20–27, 2014.

[60] F. Mohammad, T. Mondal, N. Guseva, G. K. Pandey, and C. Kanduri, "Kcnq1ot1 noncoding RNA mediates transcriptional gene silencing by interacting with Dnmt1," *Development (Cambridge, England)*, vol. 137, no. 15, pp. 2493–2499, 2010.

[61] Q. Li, Z. Su, X. Xu et al., "AS1DHRS4, a head-to-head natural antisense transcript, silences the DHRS4 gene cluster in cis and trans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 35, pp. 14110–14115, 2012.

[62] C. R. Merry, M. E. Forrest, J. N. Sabers et al., "DNMT1-associated long non-coding RNAs regulate global gene expression and DNA methylation in colon cancer," *Human Molecular Genetics*, vol. 24, no. 21, pp. 6240–6253, 2015.

[63] R. S. Lee and C. W. Roberts, "Linking the SWI/SNF complex to prostate cancer," *Nature Genetics*, vol. 45, no. 11, pp. 1268–1269, 2013.

[64] K. Arab, Y. J. Park, A. M. Lindroth et al., "Long noncoding RNA TARID directs demethylation and activation of the tumor suppressor TCF21 via GADD45A," *Molecular Cell*, vol. 55, no. 4, pp. 604–614, 2014.

[65] P. Monnier, C. Martinet, J. Pontis, I. Stancheva, S. Ait-Si-Ali, and L. Dandolo, "H19 lncRNA controls gene expression of the imprinted gene network by recruiting MBD1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20693–20698, 2013.

[66] J. R. Prensner, M. K. Iyer, A. Sahu et al., "The long noncoding RNA SChLAP1 promotes aggressive prostate cancer and antagonizes the SWI/SNF complex," *Nature Genetics*, vol. 45, no. 11, pp. 1392–1398, 2013.

[67] Y. Tang, J. Wang, Y. Lian et al., "Linking long non-coding RNAs and SWI/SNF complexes to chromatin remodeling in cancer," *Molecular Cancer*, vol. 16, no. 1, p. 42, 2017.

[68] F. Yang, X. Deng, W. Ma et al., "The lncRNA Firre anchors the inactive X chromosome to the nucleolus by binding CTCF and maintains H3K27me3 methylation," *Genome Biology*, vol. 16, no. 1, p. 52, 2015.

[69] K. C. Wang, Y. W. Yang, B. Liu et al., "A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression," *Nature*, vol. 472, no. 7341, pp. 120–124, 2011.

[70] F. Lai, U. A. Orom, M. Cesaroni et al., "Activating RNAs associate with mediator to enhance chromatin architecture and transcription," *Nature*, vol. 494, no. 7438, pp. 497–501, 2013.

[71] J. F. Xiang, Q. F. Yin, T. Chen et al., "Human colorectal cancer-specific CCAT1-L lncRNA regulates long-range chromatin interactions at the MYC locus," *Cell Research*, vol. 24, no. 5, pp. 513–531, 2014.

[72] J. C. Akers, D. Gonda, R. Kim, B. S. Carter, and C. C. Chen, "Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies," *Journal of Neuro-Oncology*, vol. 113, no. 1, pp. 1–11, 2013.

[73] D. A. Sartori and D. W. Chan, "Biomarkers in prostate cancer: what's new?" *Current Opinion in Oncology*, vol. 26, no. 3, pp. 259–264, 2014.

[74] H. Tang, Z. Wu, J. Zhang, and B. Su, "Salivary lncRNA as a potential marker for oral squamous cell carcinoma diagnosis," *Molecular Medicine Reports*, vol. 7, no. 3, pp. 761–766, 2013.

[75] E. M. Reis and S. Verjovski-Almeida, "Perspectives of long non-coding RNAs in cancer diagnostics," *Frontiers in Genetics*, vol. 3, no. 32, p. 32, 2012.

[76] Y. Shao, M. Ye, X. Jiang et al., "Gastric juice long noncoding RNA used as a tumor marker for screening gastric cancer," *Cancer*, vol. 120, no. 21, pp. 3320–3328, 2014.

[77] A. Silva, M. Bullock, and G. Calin, "The clinical relevance of long non-coding RNAs in cancer," *Cancer*, vol. 7, no. 4, pp. 2169–2182, 2015.

[78] P. Ji, S. Diederichs, W. Wang et al., "MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer," *Oncogene*, vol. 22, no. 39, pp. 8031–8041, 2003.

[79] Y. Fradet, F. Saad, A. Aprikian et al., "uPM3, a new molecular urine test for the detection of prostate cancer," *Urology*, vol. 64, no. 2, pp. 311–315, 2004, discussion 315-316.

[80] M. Tinzl, M. Marberger, S. Horvath, and C. Chypre, "DD3PCA3 RNA analysis in urine—a new perspective for detecting prostate cancer," *European Urology*, vol. 46, no. 2, pp. 182–186, 2004, discussion 187.

[81] S. B. Shappell, "Clinical utility of prostate carcinoma molecular diagnostic tests," *Reviews in Urology*, vol. 10, no. 1, pp. 44–69, 2008.

[82] M. Rasool, A. Malik, S. Zahid et al., "Non-coding RNAs in cancer diagnosis and therapy," *Non-coding RNA Research*, vol. 1, no. 1, pp. 69–76, 2016.

[83] X. Zhou, C. Yin, Y. Dang, F. Ye, and G. Zhang, "Identification of the long non-coding RNA H19 in plasma as a novel biomarker for diagnosis of gastric cancer," *Scientific Reports*, vol. 5, article 11516, 2015.

[84] S. Ren, F. Wang, J. Shen et al., "Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer," *European Journal of Cancer (Oxford, England : 1990)*, vol. 49, no. 13, pp. 2949–2959, 2013.

[85] B. Hu, H. Yang, and H. Yang, "Diagnostic value of urine prostate cancer antigen 3 test using a cutoff value of 35 mug/L in patients with prostate cancer," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, no. 9, pp. 8573–8580, 2014.

[86] T. Arita, D. Ichikawa, H. Konishi et al., "Circulating long non-coding RNAs in plasma of patients with gastric cancer," *Anticancer Research*, vol. 33, no. 8, pp. 3185–3193, 2013.

[87] K. Trajkovic, C. Hsu, S. Chiantia et al., "Ceramide triggers budding of exosome vesicles into multivesicular endosomes," *Science (New York, N.Y.)*, vol. 319, no. 5867, pp. 1244–1247, 2008.

[88] R. M. Johnstone, "Exosomes biological significance: a concise review," *Blood Cells, Molecules & Diseases*, vol. 36, no. 2, pp. 315–321, 2006.

[89] X. Huang, T. Yuan, M. Tschannen et al., "Characterization of human plasma-derived exosomal RNAs by deep sequencing," *BMC Genomics*, vol. 14, no. 1, p. 319, 2013.

[90] Q. Li, Y. Shao, X. Zhang et al., "Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 36, no. 3, pp. 2007–2012, 2015.

[91] J. D. Arroyo, J. R. Chevillet, E. M. Kroh et al., "Argonaute2 complexes carry a population of circulating microRNAs

independent of vesicles in human plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 12, pp. 5003–5008, 2011.

[92] K. Wang, S. Zhang, J. Weber, D. Baxter, and D. J. Galas, "Export of microRNAs and microRNA-protective protein by mammalian cells," *Nucleic Acids Research*, vol. 38, no. 20, pp. 7248–7259, 2010.

[93] P. Qi, X. Zhou, and X. Du, "Circulating long non-coding RNAs in cancer: current status and future perspectives," *Molecular Cancer*, vol. 15, no. 1, p. 39, 2016.

[94] J. L. Huang, L. Zheng, Y. W. Hu, and Q. Wang, "Characteristics of long non-coding RNA and its relation to hepatocellular carcinoma," *Carcinogenesis*, vol. 35, no. 3, pp. 507–514, 2014.

[95] G. L. Lee, A. Dobi, and S. Srivastava, "Prostate cancer: diagnostic performance of the PCA3 urine test," *Nature Reviews. Urology*, vol. 8, no. 3, pp. 123-124, 2011.

[96] S. B. Shappell, J. Fulmer, D. Arguello, B. S. Wright, J. R. Oppenheimer, and M. J. Putzi, "PCA3 urine mRNA testing for prostate carcinoma: patterns of use by community urologists and assay performance in reference laboratory setting," *Urology*, vol. 73, no. 2, pp. 363–368, 2009.

[97] D. Hessels, J. M. Klein Gunnewiek, I. van Oort et al., "DD3(PCA3)-based molecular urine analysis for the diagnosis of prostate cancer," *European Urology*, vol. 44, no. 1, pp. 8–15, 2003, discussion 15-16.

[98] W. J. Xue, X. L. Ying, J. H. Jiang, and Y. H. Xu, "Prostate cancer antigen 3 as a biomarker in the urine for prostate cancer diagnosis: a meta-analysis," *Journal of Cancer Research and Therapeutics*, vol. 10, Supplement, pp. C218–C221, 2014.

[99] R. Merola, L. Tomao, A. Antenucci et al., "PCA3 in prostate cancer and tumor aggressiveness detection on 407 high-risk patients: a National Cancer Institute experience," *Journal of Experimental & Clinical Cancer Research : CR*, vol. 34, no. 1, p. 15, 2015.

[100] K. K. Chevli, M. Duff, P. Walter et al., "Urinary PCA3 as a predictor of prostate cancer in a cohort of 3,073 men undergoing initial prostate biopsy," *The Journal of Urology*, vol. 191, no. 6, pp. 1743–1748, 2014.

[101] M. Svoboda, J. Slyskova, M. Schneiderova et al., "HOTAIR long non-coding RNA is a negative prognostic factor not only in primary tumors, but also in the blood of colorectal cancer patients," *Carcinogenesis*, vol. 35, no. 7, pp. 1510–1515, 2014.

[102] K. Panzitt, M. M. Tschernatsch, C. Guelly et al., "Characterization of HULC, a novel gene with striking up-regulation in hepatocellular carcinoma, as noncoding RNA," *Gastroenterology*, vol. 132, no. 1, pp. 330–342, 2007.

[103] H. Xie, H. Ma, and D. Zhou, "Plasma HULC as a promising novel biomarker for the detection of hepatocellular carcinoma," *BioMed Research International*, vol. 2013, Article ID 136106, 5 pages, 2013.

[104] Y. Zhao, Q. Guo, J. Chen, J. Hu, S. Wang, and Y. Sun, "Role of long non-coding RNA HULC in cell proliferation, apoptosis and tumor metastasis of gastric cancer: a clinical and in vitro investigation," *Oncology Reports*, vol. 31, no. 1, pp. 358–364, 2014.

[105] F. Guo, F. Yu, J. Wang et al., "Expression of MALAT1 in the peripheral whole blood of patients with lung cancer," *Biomedical Reports*, vol. 3, no. 3, pp. 309–312, 2015.

[106] D. G. Weber, G. Johnen, S. Casjens et al., "Evaluation of long noncoding RNA MALAT1 as a candidate blood-based biomarker for the diagnosis of non-small cell lung cancer," *BMC Research Notes*, vol. 6, no. 1, p. 518, 2013.

[107] F. Wang, S. Ren, R. Chen et al., "Development and prospective multicenter evaluation of the long noncoding RNA MALAT-1 as a diagnostic urinary biomarker for prostate cancer," *Oncotarget*, vol. 5, no. 22, pp. 11091–11102, 2014.

[108] Q. Pang, J. Ge, Y. Shao et al., "Increased expression of long intergenic non-coding RNA LINC00152 in gastric cancer and its clinical significance," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, no. 6, pp. 5441–5447, 2014.

[109] J. Tang, R. Jiang, L. Deng, X. Zhang, K. Wang, and B. Sun, "Circulation long non-coding RNAs act as biomarkers for predicting tumorigenesis and metastasis in hepatocellular carcinoma," *Oncotarget*, vol. 6, no. 6, pp. 4505–4515, 2015.

[110] X. S. Wang, Z. Zhang, H. C. Wang et al., "Rapid identification of UCA1 as a very sensitive and specific unique marker for human bladder carcinoma," *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, vol. 12, no. 16, pp. 4851–4858, 2006.

[111] A. R. Dallosso, A. L. Hancock, S. Malik et al., "Alternately spliced WT1 antisense transcripts interact with WT1 sense RNA and show epigenetic and splicing defects in cancer," *RNA (New York, N.Y.)*, vol. 13, no. 12, pp. 2287–2299, 2007.

[112] Y. Yao, J. Ma, Y. Xue et al., "Knockdown of long non-coding RNA XIST exerts tumor-suppressive functions in human glioblastoma stem cells by up-regulating miR-152," *Cancer Letters*, vol. 359, no. 1, pp. 75–86, 2015.

[113] Y. Wang, Y. Wang, J. Li, Y. Zhang, H. Yin, and B. Han, "CRNDE, a long-noncoding RNA, promotes glioma cell growth and invasion through mTOR signaling," *Cancer Letters*, vol. 367, no. 2, pp. 122–128, 2015.

[114] J. Ma, P. Wang, Y. Yao et al., "Knockdown of long non-coding RNA MALAT1 increases the blood-tumor barrier permeability by up-regulating miR-140," *Biochimica et Biophysica Acta*, vol. 1859, no. 2, pp. 324–338, 2016.

[115] M. H. Yang, Z. Y. Hu, C. Xu et al., "MALAT1 promotes colorectal cancer cell proliferation/migration/invasion via PRKA kinase anchor protein 9," *Biochimica et Biophysica Acta*, vol. 1852, no. 1, pp. 166–174, 2015.

[116] N. Huang, Y. Chi, J. Xue et al., "Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) interacts with estrogen receptor and predicted poor survival in breast cancer," *Oncotarget*, vol. 7, no. 25, pp. 37957–37965, 2016.

[117] P. Malakar, A. Shilo, A. Mogilevsky et al., "Long noncoding RNA MALAT1 promotes hepatocellular carcinoma development by SRSF1 upregulation and mTOR activation," *Cancer Research*, vol. 77, no. 5, pp. 1155–1167, 2017.

[118] K. Yamada, J. Kano, H. Tsunoda et al., "Phenotypic characterization of endometrial stromal sarcoma of the uterus," *Cancer Science*, vol. 97, no. 2, pp. 106–112, 2006.

[119] J. M. Silva, N. J. Boczek, M. W. Berres, X. Ma, and D. I. Smith, "LSINCT5 is over expressed in breast and ovarian cancer and affects cellular proliferation," *RNA Biology*, vol. 8, no. 3, pp. 496–505, 2011.

[120] H. Li, L. Zhu, L. Xu et al., "Long noncoding RNA linc00617 exhibits oncogenic activity in breast cancer," *Molecular Carcinogenesis*, vol. 56, no. 1, pp. 3–17, 2017.

[121] N. Xu, F. Chen, F. Wang et al., "Clinical significance of high expression of circulating serum lncRNA RP11-445H22.4 in breast cancer patients: a Chinese population-based study," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 36, no. 10, pp. 7659–7665, 2015.

[122] A. Iacoangeli, Y. Lin, E. J. Morley et al., "BC200 RNA in invasive and preinvasive breast cancer," *Carcinogenesis*, vol. 25, no. 11, pp. 2125–2133, 2004.

[123] M. Yang, X. Zhai, B. Xia, Y. Wang, and G. Lou, "Long non-coding RNA CCHE1 promotes cervical cancer cell proliferation via upregulating PCNA," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 36, no. 10, pp. 7615–7622, 2015.

[124] Y. S. Tong, X. W. Wang, X. L. Zhou et al., "Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma," *Molecular Cancer*, vol. 14, no. 1, p. 3, 2015.

[125] X. Ge, Y. Chen, X. Liao et al., "Overexpression of long noncoding RNA PCAT-1 is a novel biomarker of poor prognosis in patients with colorectal cancer," *Medical Oncology (Northwood, London, England)*, vol. 30, no. 2, p. 588, 2013.

[126] J. R. Prensner, W. Chen, S. Han et al., "The long non-coding RNA PCAT-1 promotes prostate cancer cell proliferation through cMyc," *Neoplasia (New York, N.Y.)*, vol. 16, no. 11, pp. 900–908, 2014.

[127] W. Wang, X. He, Z. Zheng et al., "Serum HOTAIR as a novel diagnostic biomarker for esophageal squamous cell carcinoma," *Molecular Cancer*, vol. 16, no. 1, p. 75, 2017.

[128] X. H. Liu, Z. L. Liu, M. Sun, J. Liu, Z. X. Wang, and W. De, "The long non-coding RNA HOTAIR indicates a poor prognosis and promotes metastasis in non-small cell lung cancer," *BMC Cancer*, vol. 13, no. 1, p. 464, 2013.

[129] M. Lee, H. J. Kim, S. W. Kim et al., "The long non-coding RNA HOTAIR increases tumour growth and invasion in cervical cancer by targeting the Notch pathway," *Oncotarget*, vol. 7, no. 28, pp. 44558–44571, 2016.

[130] K. Kim, I. Jutooru, G. Chadalapaka et al., "HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer," *Oncogene*, vol. 32, no. 13, pp. 1616–1625, 2013.

[131] Y. Wu, L. Zhang, L. Zhang et al., "Long non-coding RNA HOTAIR promotes tumor cell invasion and metastasis by recruiting EZH2 and repressing E-cadherin in oral squamous cell carcinoma," *International Journal of Oncology*, vol. 46, no. 6, pp. 2586–2594, 2015.

[132] Y. J. Geng, S. L. Xie, Q. Li, J. Ma, and G. Y. Wang, "Large intervening non-coding RNA HOTAIR is associated with hepatocellular carcinoma progression," *The Journal of International Medical Research*, vol. 39, no. 6, pp. 2119–2128, 2011.

[133] J. X. Zhang, L. Han, Z. S. Bao et al., "HOTAIR, a cell cycle-associated long noncoding RNA and a strong predictor of survival, is preferentially expressed in classical and mesenchymal glioma," *Neuro-Oncology*, vol. 15, no. 12, pp. 1595–1603, 2013.

[134] J. Ke, Y. L. Yao, J. Zheng et al., "Knockdown of long noncoding RNA HOTAIR inhibits malignant biological behaviors of human glioma cells via modulation of miR-326," *Oncotarget*, vol. 6, no. 26, pp. 21934–21949, 2015.

[135] M. Qiu, Y. Xu, X. Yang et al., "CCAT2 is a lung adenocarcinoma-specific long non-coding RNA and promotes invasion of non-small cell lung cancer," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, no. 6, pp. 5375–5380, 2014.

[136] H. Ling, R. Spizzo, Y. Atlasi et al., "CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer," *Genome Research*, vol. 23, no. 9, pp. 1446–1461, 2013.

[137] L. Wu, L. Jin, W. Zhang, and L. Zhang, "Roles of long non-coding RNA CCAT2 in cervical cancer cell growth and apoptosis," *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, vol. 22, pp. 875–879, 2016.

[138] M. D. Xu, P. Qi, W. W. Weng et al., "Long non-coding RNA LSINCT5 predicts negative prognosis and exhibits oncogenic activity in gastric cancer," *Medicine*, vol. 93, no. 28, article e303, 2014.

[139] Q. Wang, J. Zhang, Y. Liu et al., "A novel cell cycle-associated lncRNA, HOXA11-AS, is transcribed from the 5-prime end of the HOXA transcript and is a biomarker of progression in glioma," *Cancer Letters*, vol. 373, no. 2, pp. 251–259, 2016.

[140] H. Guo, L. Wu, Q. Yang, M. Ye, and X. Zhu, "Functional linc-POU3F3 is overexpressed and contributes to tumorigenesis in glioma," *Gene*, vol. 554, no. 1, pp. 114–119, 2015.

[141] C. C. Ma, Z. Xiong, G. N. Zhu et al., "Long non-coding RNA ATB promotes glioma malignancy by negatively regulating miR-200a," *Journal of Experimental & Clinical Cancer Research : CR*, vol. 35, no. 1, p. 90, 2016.

[142] L. Hu, Q. L. Lv, S. H. Chen et al., "Up-regulation of long noncoding RNA AB073614 predicts a poor prognosis in patients with glioma," *International Journal of Environmental Research and Public Health*, vol. 13, no. 4, p. 433, 2016.

[143] T. Zhou and Y. Gao, "Increased expression of LncRNA BANCR and its prognostic significance in human hepatocellular carcinoma," *World Journal of Surgical Oncology*, vol. 14, no. 1, p. 8, 2015.

[144] T. Zhang, C. Cao, D. Wu, and L. Liu, "SNHG3 correlates with malignant status and poor prognosis in hepatocellular carcinoma," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 2, pp. 2379–2385, 2016.

[145] Y. Shi, Q. Song, S. Yu, D. Hu, and X. Zhuang, "Microvascular invasion in hepatocellular carcinoma overexpression promotes cell proliferation and inhibits cell apoptosis of hepatocellular carcinoma via inhibiting miR-199a expression," *OncoTargets and Therapy*, vol. 8, pp. 2303–2310, 2015.

[146] F. Q. Nie, Q. Zhu, T. P. Xu et al., "Long non-coding RNA MVIH indicates a poor prognosis for non-small cell lung cancer and promotes cell proliferation and invasion," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, no. 8, pp. 7587–7594, 2014.

[147] N. M. White, C. R. Cabanski, J. M. Silva-Fisher, H. X. Dang, R. Govindan, and C. A. Maher, "Transcriptome sequencing reveals altered long intergenic non-coding RNAs in lung cancer," *Genome Biology*, vol. 15, no. 8, p. 429, 2014.

[148] M. Qiu, Y. Xu, J. Wang et al., "A novel lncRNA, LUADT1, promotes lung adenocarcinoma proliferation via the

epigenetic suppression of p27," *Cell Death & Disease*, vol. 6, no. 8, article e1858, 2015.

[149] Z. Zeng, H. Bo, Z. Gong et al., "AFAP1-AS1, a long noncoding RNA upregulated in lung cancer and promotes invasion and metastasis," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 1, pp. 729–737, 2016.

[150] X. Han, L. Wang, Y. Ning, S. Li, and Z. Wang, "Long noncoding RNA AFAP1-AS1 facilitates tumor growth and promotes metastasis in colorectal cancer," *Biological Research*, vol. 49, no. 1, p. 36, 2016.

[151] X. Lu, C. Zhou, R. Li et al., "Critical role for the long noncoding RNA AFAP1-AS1 in the proliferation and metastasis of hepatocellular carcinoma," *Tumour Biology*, vol. 37, no. 7, pp. 9699–9707, 2016.

[152] H. L. Luo, M. D. Huang, J. N. Guo et al., "AFAP1-AS1 is upregulated and promotes esophageal squamous cell carcinoma cell proliferation and inhibits cell apoptosis," *Cancer Medicine*, vol. 5, no. 10, pp. 2879–2885, 2016.

[153] F. Q. Nie, M. Sun, J. S. Yang et al., "Long noncoding RNA ANRIL promotes non-small cell lung cancer cell proliferation and inhibits apoptosis by silencing KLF2 and P21 expression," *Molecular Cancer Therapeutics*, vol. 14, no. 1, pp. 268–277, 2015.

[154] M. Huang, W. Chen, F. Qi et al., "Long non-coding RNA ANRIL is upregulated in hepatocellular carcinoma and regulates cell apoptosis by epigenetic silencing of KLF2," *Journal of Hematology & Oncology*, vol. 8, no. 50, 2015.

[155] H. Zhu, X. Li, Y. Song, P. Zhang, Y. Xiao, and Y. Xing, "Long non-coding RNA ANRIL is up-regulated in bladder cancer and regulates bladder cancer cell proliferation and apoptosis through the intrinsic pathway," *Biochemical and Biophysical Research Communications*, vol. 467, no. 2, pp. 223–228, 2015.

[156] W. Nie, H. J. Ge, X. Q. Yang et al., "LncRNA-UCA1 exerts oncogenic functions in non-small cell lung cancer by targeting miR-193a-3p," *Cancer Letters*, vol. 371, no. 1, pp. 99–106, 2016.

[157] Y. T. Yang, Y. F. Wang, J. Y. Lai et al., "Long non-coding RNA UCA1 contributes to the progression of oral squamous cell carcinoma by regulating the WNT/β-catenin signaling pathway," *Cancer Science*, vol. 107, no. 11, pp. 1581–1589, 2016.

[158] A. K. Srivastava, P. K. Singh, S. K. Rath, D. Dalela, M. M. Goel, and M. L. Bhatt, "Appraisal of diagnostic ability of UCA1 as a biomarker of carcinoma of the urinary bladder," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, no. 11, pp. 11435–11442, 2014.

[159] Y. Han, Y. N. Yang, H. H. Yuan et al., "UCA1, a long noncoding RNA up-regulated in colorectal cancer influences cell proliferation, apoptosis and cell cycle distribution," *Pathology*, vol. 46, no. 5, pp. 396–401, 2014.

[160] J. N. Xiao, T. H. Yan, R. M. Yu et al., "Long non-coding RNA UCA1 regulates the expression of Snail2 by miR-203 to promote hepatocellular carcinoma progression," *Journal of Cancer Research and Clinical Oncology*, vol. 143, no. 6, pp. 981–990, 2017.

[161] Y. L. Tuo, X. M. Li, and J. Luo, "Long noncoding RNA UCA1 modulates breast cancer cell growth and apoptosis through decreasing tumor suppressive miR-143," *European Review for Medical and Pharmacological Sciences*, vol. 19, no. 18, pp. 3403–3411, 2015.

[162] J. Y. Li, X. Ma, and C. B. Zhang, "Overexpression of long noncoding RNA UCA1 predicts a poor prognosis in patients with esophageal squamous cell carcinoma," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 11, pp. 7938–7944, 2014.

[163] L. Lessard, M. Liu, D. M. Marzese et al., "The CASC15 long intergenic noncoding RNA locus is involved in melanoma progression and phenotype switching," *The Journal of Investigative Dermatology*, vol. 135, no. 10, pp. 2464–2474, 2015.

[164] D. Khaitan, M. E. Dinger, J. Mazar et al., "The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion," *Cancer Research*, vol. 71, no. 11, pp. 3852–3862, 2011.

[165] J. Mazar, W. Zhao, A. M. Khalil et al., "The functional characterization of long noncoding RNA SPRY4-IT1 in human melanoma cells," *Oncotarget*, vol. 5, no. 19, pp. 8959–8969, 2014.

[166] H. Liu, Z. Lv, and E. Guo, "Knockdown of long noncoding RNA SPRY4-IT1 suppresses glioma cell proliferation, metastasis and epithelial-mesenchymal transition," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 8, pp. 9140–9146, 2015.

[167] M. Luo, Z. Li, W. Wang, Y. Zeng, Z. Liu, and J. Qiu, "Long non-coding RNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression," *Cancer Letters*, vol. 333, no. 2, pp. 213–221, 2013.

[168] D. Tan, Y. Wu, L. Hu et al., "Long noncoding RNA H19 is up-regulated in esophageal squamous cell carcinoma and promotes cell proliferation and metastasis," *Diseases of the Esophagus : Official Journal of the International Society for Diseases of the Esophagus*, vol. 30, no. 1, pp. 1–9, 2017.

[169] W. Yang, N. Ning, and X. Jin, "The lncRNA H19 promotes cell proliferation by competitively binding to miR-200a and derepressing beta-catenin expression in colorectal cancer," *BioMed Research International*, vol. 2017, Article ID 2767484, 8 pages, 2017.

[170] Y. Shi, Y. Wang, W. Luan et al., "Long non-coding RNA H19 promotes glioma cell invasion by deriving miR-675," *PloS One*, vol. 9, no. 1, article e86295, 2014.

[171] W. Peng, W. Gao, and J. Feng, "Long noncoding RNA HULC is a novel biomarker of poor prognosis in patients with pancreatic cancer," *Medical Oncology (Northwood, London, England)*, vol. 31, no. 12, p. 346, 2014.

[172] Y. Zhu, X. Zhang, L. Qi et al., "HULC long noncoding RNA silencing suppresses angiogenesis by regulating ESM-1 via the PI3K/Akt/mTOR signaling pathway in human gliomas," *Oncotarget*, vol. 7, no. 12, pp. 14429–14440, 2016.

[173] A. Ylipaa, K. Kivinummi, A. Kohvakka et al., "Transcriptome sequencing reveals PCAT5 as a novel ERG-regulated long noncoding RNA in prostate cancer," *Cancer Research*, vol. 75, no. 19, pp. 4026–4031, 2015.

[174] F. Crea, A. Watahiki, L. Quagliata et al., "Identification of a long non-coding RNA as a novel biomarker and potential therapeutic target for metastatic prostate cancer," *Oncotarget*, vol. 5, no. 3, pp. 764–774, 2014.

[175] S. Chung, H. Nakagawa, M. Uemura et al., "Association of a novel long non-coding RNA in 8q24 with prostate cancer susceptibility," *Cancer Science*, vol. 102, no. 1, pp. 245–252, 2011.

[176] C. He, B. Jiang, J. Ma, and Q. Li, "Aberrant NEAT1 expression is associated with clinical outcome in high grade glioma patients," *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 124, no. 3, pp. 169–174, 2016.

[177] L. Zhen, L. Yun-Hui, D. Hong-Yu, M. Jun, and Y. Yi-Long, "Long noncoding RNA NEAT1 promotes glioma pathogenesis by regulating miR-449b-5p/c-Met axis," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 1, pp. 673–683, 2016.

[178] S. Guo, W. Chen, Y. Luo et al., "Clinical implication of long non-coding RNA NEAT1 expression in hepatocellular carcinoma patients," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 5, pp. 5395–5402, 2015.

[179] Y. Lu, T. Li, G. Wei et al., "The long non-coding RNA NEAT1 regulates epithelial to mesenchymal transition and radioresistance in through miR-204/ZEB1 axis in nasopharyngeal carcinoma," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 9, pp. 11733–11741, 2016.

[180] Q. Zhou, J. Chen, J. Feng, and J. Wang, "Long noncoding RNA PVT1 modulates thyroid cancer cell proliferation by recruiting EZH2 and regulating thyroid-stimulating hormone receptor (TSHR)," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 3, pp. 3105–3113, 2016.

[181] R. Kong, E. B. Zhang, D. D. Yin et al., "Long noncoding RNA PVT1 indicates a poor prognosis of gastric cancer and promotes cell proliferation through epigenetically regulating p15 and p16," *Molecular Cancer*, vol. 14, no. 1, p. 82, 2015.

[182] Y. Takahashi, G. Sawada, J. Kurashige et al., "Amplification of PVT-1 is involved in poor prognosis via apoptosis inhibition in colorectal cancers," *British Journal of Cancer*, vol. 110, no. 1, pp. 164–171, 2014.

[183] R. Yan, K. Wang, R. Peng et al., "Genetic variants in lncRNA SRA and risk of breast cancer," *Oncotarget*, vol. 7, no. 16, pp. 22486–22496, 2016.

[184] P. Wang, Z. Ren, and P. Sun, "Overexpression of the long non-coding RNA MEG3 impairs in vitro glioma cell proliferation," *Journal of Cellular Biochemistry*, vol. 113, no. 6, pp. 1868–1874, 2012.

[185] J. Li, E. B. Bian, X. J. He et al., "Epigenetic repression of long non-coding RNA MEG3 mediated by DNMT1 represses the p53 pathway in gliomas," *International Journal of Oncology*, vol. 48, no. 2, pp. 723–733, 2016.

[186] M. E. Askarian-Amiri, J. Crawford, J. D. French et al., "SNORD-host RNA Zfas1 is a regulator of mammary development and a potential marker for breast cancer," *RNA (New York, N.Y.)*, vol. 17, no. 5, pp. 878–891, 2011.

[187] M. Mourtada-Maarabouni, M. R. Pickard, V. L. Hedge, F. Farzaneh, and G. T. Williams, "GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer," *Oncogene*, vol. 28, no. 2, pp. 195–208, 2009.

[188] X. Zhao, P. Wang, J. Liu et al., "Gas5 exerts tumor-suppressive functions in human glioma cells by targeting miR-222," *Molecular Therapy : The Journal of the American Society of Gene Therapy*, vol. 23, no. 12, pp. 1899–1911, 2015.

[189] J. Ding, B. Lu, J. Wang et al., "Long non-coding RNA Loc554202 induces apoptosis in colorectal cancer cells via the caspase cleavage cascades," *Journal of Experimental & Clinical Cancer Research : CR*, vol. 34, no. 1, article 100, 2015.

[190] L. Dong, P. Qi, M. D. Xu et al., "Circulating CUDR, LSINCT-5 and PTENP1 long noncoding RNAs in sera distinguish patients with gastric cancer from healthy controls," *International Journal of Cancer*, vol. 137, no. 5, pp. 1128–1135, 2015.

[191] L. Poliseno, L. Salmena, J. Zhang, B. Carver, W. J. Haveman, and P. P. Pandolfi, "A coding-independent function of gene and pseudogene mRNAs regulates tumour biology," *Nature*, vol. 465, no. 7301, pp. 1033–1038, 2010.

[192] Z. H. Fei, X. J. Yu, M. Zhou, H. F. Su, Z. Zheng, and C. Y. Xie, "Upregulated expression of long non-coding RNA LINC00982 regulates cell proliferation and its clinical relevance in patients with gastric cancer," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 2, pp. 1983–1993, 2016.

[193] X. Qin, J. Yao, P. Geng, X. Fu, J. Xue, and Z. Zhang, "LncRNA TSLC1-AS1 is a novel tumor suppressor in glioma," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 6, pp. 3065–3072, 2014.

[194] J. Yao, B. Zhou, J. Zhang et al., "A new tumor suppressor LncRNA ADAMTS9-AS2 is regulated by DNMT1 and inhibits migration of glioma cells," *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, no. 8, pp. 7935–7944, 2014.

[195] H. Yue, J. Zhu, S. Xie, F. Li, and Q. Xu, "MDC1-AS, an antisense long noncoding RNA, regulates cell proliferation of glioma," *Biomedicine & Pharmacotherapy*, vol. 81, pp. 203–209, 2016.

[196] J. Li, M. Zhang, G. An, and Q. Ma, "LncRNA TUG1 acts as a tumor suppressor in human glioma by promoting cell apoptosis," *Experimental Biology and Medicine (Maywood, N.J.)*, vol. 241, no. 6, pp. 644–649, 2016.

[197] S. Feng, J. Yao, Y. Chen et al., "Expression and functional role of reprogramming-related long noncoding RNA (lincRNA-ROR) in glioma," *Journal of Molecular Neuroscience : MN*, vol. 56, no. 3, pp. 623–630, 2015.

[198] P. Wang, Y. H. Liu, Y. L. Yao et al., "Long non-coding RNA CASC2 suppresses malignancy in human gliomas by miR-21," *Cellular Signalling*, vol. 27, no. 2, pp. 275–282, 2015.

[199] C. C. Zhou, F. Yang, S. X. Yuan et al., "Systemic genome screening identifies the outcome associated focal loss of long noncoding RNA PRAL in hepatocellular carcinoma," *Hepatology (Baltimore, Md.)*, vol. 63, no. 3, pp. 850–863, 2016.

[200] H. He, R. Nagy, S. Liyanarachchi et al., "A susceptibility locus for papillary thyroid carcinoma on chromosome 8q24," *Cancer Research*, vol. 69, no. 2, pp. 625–631, 2009.

[201] J. H. Yang, J. H. Li, S. Jiang, H. Zhou, and L. H. Qu, "ChIPBase: a database for decoding the transcriptional regulation of long non-coding RNA and microRNA genes from ChIP-Seq data," *Nucleic Acids Research*, vol. 41, no. Database issue, pp. D177–D187, 2013.

[202] T. Weirick, D. John, S. Dimmeler, and S. Uchida, "C-It-Loci: a knowledge database for tissue-enriched loci," *Bioinformatics (Oxford, England)*, vol. 31, no. 21, pp. 3537–3543, 2015.

[203] Z. Zhao, J. Bai, A. Wu et al., "Co-LncRNA: investigating the lncRNA combinatorial effects in GO annotations and KEGG pathways based on human RNA-Seq data," *Database : The Journal of Biological Databases and Curation*, vol. 2015, article bav082, 2015.

[204] M. D. Paraskevopoulou, G. Georgakilas, N. Kostoulas et al., "DIANA-LncBase: experimentally verified and computationally predicted microRNA targets on long non-coding RNAs," *Nucleic Acids Research*, vol. 41, Database issue, pp. D239–D245, 2013.

[205] M. D. Paraskevopoulou, I. S. Vlachos, D. Karagkouni et al., "DIANA-LncBase v2: indexing microRNA targets on non-coding transcripts," *Nucleic Acids Research*, vol. 44, no. D1, pp. D231–D238, 2016.

[206] K. Liu, Z. Yan, Y. Li, and Z. Sun, "Linc2GO: a human LincRNA function annotation resource based on ceRNA hypothesis," *Bioinformatics (Oxford, England)*, vol. 29, no. 17, pp. 2221–2222, 2013.

[207] S. Ning, J. Zhang, P. Wang et al., "Lnc2Cancer: a manually curated database of experimentally supported lncRNAs associated with various human cancers," *Nucleic Acids Research*, vol. 44, no. D1, pp. D980–D985, 2016.

[208] P. Wang, S. Ning, Y. Zhang et al., "Identification of lncRNA-associated competing triplets reveals global patterns and prognostic markers for cancer," *Nucleic Acids Research*, vol. 43, no. 7, pp. 3478–3489, 2015.

[209] P. J. Volders, K. Helsens, X. Wang et al., "LNCipedia: a database for annotated human lncRNA transcript sequences and structures," *Nucleic Acids Research*, vol. 41, Database issue, pp. D246–D251, 2013.

[210] P. J. Volders, K. Verheggen, G. Menschaert et al., "An update on LNCipedia: a database for annotated human lncRNA sequences," *Nucleic Acids Research*, vol. 43, no. 8, pp. 4363–4364, 2015.

[211] S. Chakraborty, A. Deb, R. K. Maji, S. Saha, and Z. Ghosh, "LncRBase: an enriched resource for lncRNA information," *PloS One*, vol. 9, no. 9, article e108010, 2014.

[212] Q. Jiang, R. Ma, J. Wang et al., "LncRNA2Function: a comprehensive resource for functional investigation of human lncRNAs based on RNA-seq data," *BMC Genomics*, vol. 16, Supplement 3, p. S2, 2015.

[213] P. P. Amaral, M. B. Clark, D. K. Gascoigne, M. E. Dinger, and J. S. Mattick, "lncRNAdb: a reference database for long noncoding RNAs," *Nucleic Acids Research*, vol. 39, Database issue, pp. D146–D151, 2011.

[214] X. C. Quek, D. W. Thomson, J. L. Maag et al., "lncRNAdb v2.0: expanding the reference database for functional long noncoding RNAs," *Nucleic Acids Research*, vol. 43, Database issue, pp. D168–D173, 2015.

[215] G. Chen, Z. Wang, D. Wang et al., "LncRNADisease: a database for long-non-coding RNA-associated diseases," *Nucleic Acids Research*, vol. 41, Database issue, pp. D983–D986, 2013.

[216] J. Gong, W. Liu, J. Zhang, X. Miao, and A. Y. Guo, "lncRNASNP: a database of SNPs in lncRNAs and their potential functions in human and mouse," *Nucleic Acids Research*, vol. 43, Database issue, pp. D181–D186, 2015.

[217] D. Bhartiya, K. Pal, S. Ghosh et al., "lncRNome: a comprehensive knowledgebase of human long noncoding RNAs," *Database : The Journal of Biological Databases and Curation*, vol. 2013, p. bat034, 2013.

[218] A. Jeggari, D. S. Marks, and E. Larsson, "miRcode: a map of putative microRNA target sites in the long non-coding transcriptome," *Bioinformatics (Oxford, England)*, vol. 28, no. 15, pp. 2062-2063, 2012.

[219] C. Liu, B. Bai, G. Skogerbo et al., "NONCODE: an integrated knowledge database of non-coding RNAs," *Nucleic Acids Research*, vol. 33, Database issue, pp. D112–D115, 2005.

[220] Y. Zhao, H. Li, S. Fang et al., "NONCODE 2016: an informative and valuable data source of long non-coding RNAs," *Nucleic Acids Research*, vol. 44, no. D1, pp. D203–D208, 2016.

[221] J. H. Yang, J. H. Li, P. Shao, H. Zhou, Y. Q. Chen, and L. H. Qu, "starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data," *Nucleic Acids Research*, vol. 39, Database issue, pp. D202–D209, 2011.

[222] J. H. Li, S. Liu, H. Zhou, L. H. Qu, and J. H. Yang, "starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data," *Nucleic Acids Research*, vol. 42, Database issue, pp. D92–D97, 2014.

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Review Article

Can Mitochondria DNA Provide a Novel Biomarker for Evaluating the Risk and Prognosis of Colorectal Cancer?

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Colorectal cancer (CRC) was one of the most frequent cancers worldwide. Accurate risk and prognosis evaluation could obtain better quality of life and longer survival time for the patients. Current research hotspot was focus on the gene biomarker to evaluate the risk and prognosis. Mitochondrion contains its own DNA and regulates self-replicating so that it can be as a candidate biomarker for evaluating the risk and prognosis of colorectal cancer. But there were already huge controversies on this issue. The review was to summarize current viewpoints of the controversial issues and described our understanding from the four aspects including mtDNA copy number, mitochondrial displacement loop, mtDNA variation, and mtDNA microsatellite instability, wishing the summary of the mtDNA in colorectal cancer could provide a meaningful reference or a valuable direction in the future studies.

1. Introduction

Colorectal cancer (CRC) was one of the common types of cancer with a high prevalence of cancer-related morbidity and mortality. Early diagnosis and accurate prognosis evaluation were conducive to reducing the negative health impact. Despite the continuous progress in diagnostic and therapeutic methods, it was still of great significance to seek for the novel biomarkers to evaluate the risk and prognosis of colorectal cancer. The promising biomarkers for this purpose were genes [1], noncoding RNAs [2, 3], proteins [4], gut microorganism [5], and so on. Seeking for more sensitive and accurate biomarker to evaluate the risk and prognosis of colorectal cancer was still striving on the way.

Organelles are the major components of the cells and play an important role in the occurrence and development of cancer cells. The variation of organelles deserves to be the biomarkers to evaluate the risk and prognosis of cancers. Mitochondria, as a vital organelle in almost all eukaryotic cells, have two major functions: oxidative phosphorylation and ATP production [6, 7]. Accumulating evidences suggested the mitochondria played a key role in the cell proliferation [8], cell apoptosis [9], and aging [10]. More, the mtDNA seemed to be involved in a wide range of diseases such as diabetes [11], obesity [12], AIDS [13], and cancers.

Mitochondrion contained its own DNA and regulated self-replicating. MtDNA (mitochondria DNA) was a circular, double-stranded DNA molecule. The outside loop was heavy strand (H-strand) and the inside was light strand (L-strand). The H-strand and the L-strand contained 28 and 9 genes, respectively. The mtDNA genes were lacking of intron coding regions and the displacement loop (D-loop) was the only one significant noncoding region of the mtDNA. The copies and point mutations of mtDNA varied with different individuals, organs ages, and diseases [14, 15]. Therefore, mtDNA copy number, mitochondrial displacement loop, and mtDNA variation play an important role in many mitochondrion-associated diseases.

The biological characteristics of mtDNA were as follows: (1) Matrilineal inheritance: matrilineal inheritance was a kind of genetic phenomenon that the genetic information passed down only through the female line. The mtDNA of fathers could not be transferred to the next generation, because the mtDNA of sperm could be specifically recognized and degraded by the ubiquitin hydrolases of ovum. (2) Heterogeneity: the mutations of mtDNA often occurred in the different mitochondria attributing to the mtDNA without the allelic gene like the nDNA (nuclear DNA). The results leaded to both of the mutant type and wild type in the same cells or tissues. (3) Threshold effect: a small number of mutant-type

mtDNA in the cells could not affect the normal structure or function of the tissues because of the compensation action of wild-type mtDNA. The structure or function of the bodies or tissues could be affected only when the quantity of mutant-type mtDNA exceeded the threshold level [16, 17]. (4) Random distribution: the number and types of mtDNA of the somatic cells or ovum cells were randomly assigned to daughter cells when it passed to next generations [18].

An abundance of studies were concentrated on the association between the mtDNA and the CRC. But there were considerable debates on whether it could be utilized as valuable biomarkers of CRC. The goal of this review was to summarize current viewpoints of the controversial issues and to reveal new directions for future studies.

2. mtDNA Copy Number

The number of mitochondrial DNAs was different between individuals and between the tissues in human cells [19]. Changes of mitochondrial DNA (mtDNA) copy number were widely reported in CRCs. Both an increase and a decrease in mtDNA copy number had been reported. It remained controversial whether the mtDNA copy number could be utilized as valuable biomarkers to evaluate the risk and prognosis of CRCs. Here we gave a short summary on this subject.

The related literatures and research achievements of mtDNA copy number in CRCs were showed in Table 1. van Osch et al. found that mtDNA copy number was lower in CRC tissues and mtDNA copy number was lower in mutated BRAF and in microsatellite unstable (MSI) tumors, but it was higher in KRAS mutated tumors [20]. Wang et al. reported that the mtDNA copy number was lower in CRC tissues and it was correlated with lymph-node metastasis [21].

However, there were many contradictive views. Wang et al. supported that the mtDNA copy number increased in CRCs and it was a factor of poor prognosis [22]. The researches of Haja Mohideen et al. suggested that mtDNA copy number both increased and decreased in CRCs and no association of the mtDNA copy number change with OS or DFS [23]. More, latest studies in vitro showed both of p53 and TFAM expression could increase mtDNA copy number in CRC lines [24].

In the studies of free circulating mtDNA, Thyagarajan et al. investigated peripheral blood mtDNA copy number in 412 colorectal adenoma cases and 526 cancer-free controls and found there was no association between mitochondrial DNA copy number and colorectal adenomas by the analysis of unconditional logistic regression [25]. Some researchers put forward an idea about the U-shaped association between the relative mtDNA copy number in peripheral blood samples and colorectal cancer risk. Individuals with lower or higher relative mtDNA copy numbers were at increased risk of colorectal cancer [26]. In addition, Qu et al. found that when the leukocyte mtDNA content was higher, the prognosis in CRC patients was worse [27]. A prospective study found that the leukocytes mtDNA copy number among women who subsequently developed colorectal cancer was lower than that among women who remained cancer-free [28].

3. D-Loop

The D-loop (mitochondrial displacement loop) was an mtDNA noncoding region and it was as the major control region for the regulation of mitochondrial genome replication and expression as it contained the leading-strand origin of replication and the main promoter required for transcription. The entire length of D-loop was 1,124 bps according to the mitochondria database http://www.mitomap.org [29–31]. The D-loop was as the research hotspot and its complete or partial sequence had been widely investigated in CRCs. Whether the D-loop could be used to evaluate the risk and prognosis of colorectal cancer, the major point of the controversy was the D-loop mutations site and D-loop mutations frequency.

The related literatures and research results of D-loop in CRCs were showed in Table 2. Feng et al. investigated 44 colorectal cancer tissues and found the ratio of the methylation in D-loop region in colorectal cancer tissues was less than that in the noncancerous tissues [32]. Gao et al. found the similar results in 65 colorectal cancer patients [33]. Govatati et al. investigated the D-loop region of CRC patients in south Indian origin and put forward that D-loop sequence alterations were inherent risk factor for CRCs [34].

Furthermore, Bai et al. believed the minor haplotype of nucleotides 16290T in the D-loop region could be used as the biomarker to evaluate the prognosis for postoperative survival of CRCs [35]. And Legras et al. found the mutations of D310 sequence could be considered as a biomarker for early detection of CRCs [36].

Kassem et al. reported that the D-loop mutations frequency was higher in CRC and precancerous colorectal lesions [37], while Akouchekian et al. held that it was more likely to be epiphenomena [38]. But Chang et al. maintained that D-loop mutation occurred at significantly higher frequency in CRCs with p53 mutations [39]. Additionally, Chang et al. held it was not associated with prognosis of CRC patients [39], but Lièvre et al. believed that the D-loop mutation was a factor of poor prognosis in CRCs [40].

4. mtDNA Alterations

The technique of SNP (Single Nucleotide Polymorphism) was mostly used to investigate the mtDNA alterations in CRCs. The mitochondria DNA, as a biomarker for the diagnosis and prognosis of CRCs, varied from study to study. The controversy was focus on the site and frequency of mtDNA mutation in CRC.

The related literatures and research results of mtDNA alteration in CRCs were showed in Table 3. Numerous research studies showed the mtDNA mutation could be used as a biomarker for the diagnosis and prognosis of CRCs [65, 68, 69]; Chen et al. investigated 104 colorectal cancer patients in China and found the mtDNA proportion of the mtDNA 4977 bp deletion in CRC tissues was decreased [62]. And this view was supported by Dimberg et al. in Swedish and Vietnamese patients [55]. Furthermore, some mtDNA mutations such as mitochondrial subunit ND1 gene [61], G1576A (MT-RNR1) and G2975A (MT-RNR2) [70], and mitochondrial

Table 1: Association between the mtDNA copy number and the risk and prognosis in CRC.

Sample type	Findings	Potential utility	Association	Ref
Cancer, adenoma, and adjacent normal tissue from CRC patients ($n = 56$) and recurrent CRC ($n = 16$); colon mucosa samples from healthy subjects ($n = 76$).	MtDNA copy number in carcinoma tissues and adjacent tissues was lower than that in earlier resected adenoma tissues and MtDNA copy number in primary CRC tissues was lower than that in recurrent CRC tissues.	Prognosis evaluation	The association between mtDNA and survival seemed to follow an inverse U-shape with the highest HR observed in the second quintile of mtDNA copy number (HR = 1.70, 95% CI = 1.18, 2.44) compared to the first quintile.	[20]
Colorectal cancer tissues ($n = 65$) and the corresponding noncancerous tissues.	The mean relative mtDNA copy number in colorectal cancer tissues was higher than that in noncancerous tissues.	Risk evaluation	Increased in the CRC tissues.	[33]
Colorectal adenoma tissues ($n = 412$) and cancer-free controls ($n = 526$).	There was no association between logarithmically transformed relative mtDNA copy number and colorectal adenoma risk.	Risk evaluation	No association.	[25]
Colorectal cancer tissues ($n = 274$) and the corresponding noncancerous tissues.	The mtDNA copy number was increased in 60.4% of the CRC tissues. But there was no association between the mtDNA copy number and the prognosis.	Risk evaluation and prognosis evaluation	Increased in the CRC tissues but no association with the prognosis.	[23]
Colorectal cancer tissues ($n = 9$) and cancer-free controls ($n = 9$).	The mtDNA copy number was decreased in adenocarcinoma.	—	Decreased in adenocarcinoma.	[41]
Leukocyte CRC patients ($n = 598$).	Patients with high leukocyte mtDNA content showed worse overall survival (OS) and relapse-free survival (RFS).	Prognosis evaluation	Negative correlation between leukocyte mtDNA content and prognosis.	[27]
peripheral leukocytes from CRC ($n = 444$) and controls nested ($n = 1,423$).	Baseline mtDNA copy number was lower among women who subsequently developed colorectal cancer.	Risk evaluation	Lower in colorectal cancers.	[28]
Colorectal cancer tissues ($n = 60$) and the corresponding noncancerous tissues.	The mtDNA copy number was lower in CRC tissues and it was correlated with lymph-node metastasis. Patients with a lower mtDNA copy number tended to have lower 3-year survival.	Risk evaluation and prognosis evaluation	Decreased in the CRC tissues.	[42]
Colorectal cancer tissues ($n = 44$) and the corresponding noncancerous tissues.	The mtDNA copy number was increased in the CRC tissues and this increase was particularly noticeable in stages I and II.	Risk evaluation	Increased in the CRC tissues.	[43]
422 colorectal cancer cases (168 cases with prediagnostic blood and 254 cases with postdiagnostic blood) and 874 controls who were free of colorectal cancer among participants.	There was a U-shaped relationship between the relative mtDNA copy number and colorectal cancer risk. The lowest and highest quartiles of relative mtDNA copy numbers were 1.81 (1.13–2.89) and 3.40 (2.15–5.36), respectively.	Risk evaluation	U-shaped association between the relative mtDNA copy number and risk of colorectal cancer.	[26]
Colorectal cancer tissues ($n = 54$) and the corresponding noncancerous tissues.	The mtDNA copy number was increased in the CRC tissues.	Risk evaluation	Increased in the CRC tissues.	[44]
Colorectal cancer tissues ($n = 25$) and the corresponding noncancerous tissues.	The mtDNA copy number was decreased in CRCs.	Risk evaluation	Decreased in the CRC tissues.	[45]

The mitochondrial DNAs copy number was different between individuals and between the tissues in human cells. Changes of mtDNA copy number were widely reported in CRCs. The PCR was used as the most common method to detect the mtDNA copy number in the tissues. There were contradictory points of the association between the mtDNA copy number and the risk and prognosis in CRC.

A12308G in tRNA (Leu(CUN)) [53] were considered as the valuable molecular targets. Besides, some research studies suggested mtDNA mutations heralded poor outcomes and tumorigenesis [44]. But several studies showed no association between mtDNA alterations and CRC risks [52, 64, 71].

5. mtDNA Microsatellite Instability

It had been already confirmed that MSI-H (high frequency MSI), MSI-L (low frequency MSI), and MSS (microsatellite stability) in human nuclear genome were significantly

Table 2: Association between the D-loop and the risk and prognosis in CRC.

Sample type	Findings	Potential utility	Targets	Ref
CRC tissues ($n = 174$) and cancer-free controls ($n = 170$)	The frequencies of 310'C' insertion ($p = 0.0078$), T16189C ($p = 0.0097$) variants, and 310'C'ins/16189C haplotype ($p = 0.0029$) in colorectal cancer were significantly higher than that in controls.	Risk evaluation	Nucleotide positions D310 and D16189	[34]
CRC tissues ($n = 25$)	The D310 mutation was found in 8/25 (32%) CRCs.	Risk evaluation	D310	[46]
Blood samples from 152 CRC patients	The minor haplotype of nucleotides 16290T and frequent haplotype of nucleotide 16298T in the hypervariable segment 1 (HV1) region of the D-loop were associated with high survival rate of CRCs. The nucleotide site of 16290 was identified as independent predictor for CRCs (RR, 0.379; 95% CI, 0.171–0.839; $p = 0.017$).	Risk evaluation and prognosis evaluation	16290T in HV1 region of the D-loop	[47]
CRC tissues ($n = 65$) and the corresponding noncancerous tissues	The methylation rate of the D-loop region in colorectal cancer tissues was decreased in clinicopathological stages III and IV comparing with that in stages I and II.	Prognosis evaluation	The methylation rate of D-loop	[33]
121 adenomas and seven adenocarcinomas and their corresponding germinal controls	The hypervariable sequence (HV-II) in the loop (D-loop) was significantly associated with the MT-CO2 gene, which represents the early molecular events in MAP (MUTYH-associated polyposis) tumorigenesis.	Risk evaluation and prognosis evaluation	HV-II	[48]
CRC tissues ($n = 44$) and the corresponding noncancerous tissues	The D-loop of most corresponding noncancerous tissues was methylated and the percentage was 79.5%, while this percentage was much smaller than that in the tumor tissues (11.4%).	Risk evaluation	The methylation rate of D-loop	[32]
Table 4 in the reference	The rate of D-loop mutations in CRCs was higher.	Risk evaluation	D-loop mutations frequency	[37]
Colorectal adenoma tissues ($n = 40$) and cancer-free controls ($n = 150$)	The rate of D-loop mutations in CRCs was higher.	Risk evaluation	D-loop mutations frequency	[38]
CRC tissues with p53 mutation ($n = 88$) and without p53 mutation ($n = 106$)	The rate of D-loop mutations was higher in CRCs with p53 mutation.	Risk evaluation	D-loop mutations frequency	[39]
64 colorectal adenomas (larger than 10 mm) and from 36 liver metastases of 15 metastatic CRC patients.	The mitochondrial D310 mutations frequency increased in the colorectal adenomas.	Risk evaluation	D310	[36]
Colorectal cancer tissues ($n = 25$) and the corresponding noncancerous tissues	40.0% (10/25) of the colorectal cancers harbored mutation(s) in the D-loop of mtDNA.	Risk evaluation	D-loop mutations frequency	[41]
Colorectal cancer tissues ($n = 77$) and the corresponding noncancerous tissues	9% (7/77) of the colorectal cancers harbored mutation(s) in the D-loop region of mtDNA.	Risk evaluation	D-loop mutations	[49]
CRC tissues ($n = 35$) and the corresponding noncancerous tissues	Polymorphisms located in hypervariable region I (67.9%) more than that in II (32.1%) of D-loop.	Risk evaluation and prognosis evaluation	Polymorphisms in the D-loop	[50]
CRC tissues ($n = 95$) and cancer-free controls ($n = 95$)	Thirty-two (34%) CRCs and 2 persons (2%) of the cancer-free controls harbored mutations in the D310 region of D-loop.	Risk evaluation	D310	[51]

The D-loop (mitochondrial displacement loop) was an mtDNA noncoding region and it was as the major control region for the regulation of mitochondrial genome replication and expression. The rate of D-loop mutations, the site of D-loop mutations, and the methylation rate of D-loop were investigated in CRCs. The table has summarized the current main points.

Table 3: Association between the mtDNA mutation and the risk and prognosis in CRC.

Sample type	Findings	Potential utility	Targets	Ref
CRC tissues ($n = 50$) and the corresponding noncancerous tissues. Control group comprised the blood samples from healthy persons ($n = 100$).	There was no association between the CAG repeat variants in the POLG gene and colorectal cancer risk.	Risk evaluation	CAG repeat variability in the POLG gene	[52]
CRC tissues ($n = 30$), control group comprised the blood samples from healthy persons ($n = 100$).	The A12308G, a polymorphic mutation in V-loop tRNA (Leu(CUN)), was found in 6 colorectal tumor tissues and 3 healthy controls.	Risk evaluation	A12308G alteration in tRNALeu (CUN)	[53]
60 Vietnamese and 138 Japanese CRCs tissues.	The frequency of mtDNA mutations in the Vietnamese CRCs was higher than that in the Japanese CRCs (19 of 44 [43%] versus 11 of 133 [9%], $p < 0.001$).	Risk evaluation	mtDNA mutations frequency	[54]
CRC tissues from 105 Swedish and 88 Vietnamese patients and the corresponding noncancerous tissues.	The mtDNA 4977 bp deletion was more frequent in normal tissues comparing with paired cancer tissues.	Risk evaluation	mtDNA 4977 bp deletion	[55]
CRC tissues ($n = 21$) and the corresponding noncancerous tissues.	The mtDNA mutation frequency in the CRC tissues was decreased comparing with adjacent nontumor tissues.	Risk evaluation	mtDNA mutations frequency	[56]
CRC tissues ($n = 54$) and the corresponding noncancerous tissues.	mtDNA haplogroup B4 was associated with colorectal cancer risk and poor outcomes.	Risk evaluation and prognosis evaluation	mtDNA haplogroup B4	[44]
Colon cancer ($n = 86$), rectal cancer ($n = 43$), and the corresponding noncancerous tissues.	Nonsynonymous mtDNA mutation was found in 57% of colon and rectal cancer	Risk evaluation	mtDNA mutations frequency	[57]
Three tissues (cancerous, paracancerous, and normal tissues), respectively, from 20 patients.	The frequency of mtDNA mutations: cancerous > paracancerous > normal tissues.	Risk evaluation	mtDNA mutations frequency	[58]
Hyperplastic polyps ($n = 25$), serrated adenomas ($n = 32$), traditional serrated adenomas ($n = 19$), and CRCs tissues ($n = 138$).	The mtDNA mutations frequency in carcinomas was not significantly higher than that in hyperplastic polyps and serrated adenomas.	Risk evaluation	mtDNA mutations frequency	[59]
CRC tissues ($n = 30$) and the corresponding noncancerous tissues.	T4216C mutation was in 8/30 CRC patients.	Risk evaluation	T4216C mutation	[60]
CRC tissues ($n = 30$) and the corresponding noncancerous tissues. Blood samples were from 25 healthy people.	The mtND1 gene mutations and polymorphisms were in 11 (45.8%) and 13 (54.2%) CRC tissues, respectively.	Risk evaluation	Mitochondrial subunit ND1 (mtND1)	[61]
CRC tissues ($n = 104$) and the corresponding noncancerous tissues.	The 4,977 bp deletion level decreased with the advancing of cancer.	Risk evaluation and prognosis evaluation	4,977 bp deletion in the major arch of the mitochondrial genome	[62]
Nuclear microsatellite instability in 38 rectal carcinomas and 25 sigmoid carcinomas.	ND1 microsatellite sequence alterations were detected in 2.6% rectal carcinomas. ND5 microsatellite sequence alterations were detected in 5.3% rectal carcinomas and 8% sigmoid carcinomas.	Risk evaluation	ND1 and ND5	[63]
2854 CRC cases and 2822 controls.	Five variants showed association with colon cancer. Three variants were associated with risk of CRC for MSI cases, with the strongest association for T4562C.	Risk evaluation	The T4562C sites	[64]

The mtDNA mutations frequency and mutations sites were investigated to explore the association between the mtDNA mutation and the risk and prognosis in CRC. But the association between mtDNA mutation and CRCs varied from study to study.

Table 4: Association between the mtDNA microsatellite instability and the risk and prognosis in CRC.

Sample type	Findings	Potential utility	Ref
CRC tissues ($n = 100$) and the corresponding noncancerous tissues.	The mtMSI was found in 30% of CRCs and it was associated with the poor prognosis.	Risk evaluation and prognosis evaluation	[65]
83 CRC tissues with a MSI tumor (including 39 patients with Lynch syndrome) and in 99mCRC patients with a microsatellite stable (MSS) tumor.	The mtMSI was high in mCRC patients with both MSI and MSS tumors, but no correlation with prognosis.	Risk evaluation and prognosis evaluation	[51]
The microdissected cancer epithelia and adjacent stromas of 48 sporadic CRCs.	The stromal mtMSI had no association with stromal nMSI or epithelial mtMSI.	Risk evaluation	[66]
CRC tissues ($n = 35$) and the corresponding noncancerous tissues.	mtMSI [310'C' insertion ($p = 0.00001$) and T16189C ($p = 0.0007$)] was increased in the CRC tissues.	Risk evaluation	[67]

Recent studies showed that nuclear genome microsatellite instability was the significant predictor of prognosis CRCs. But the association between the mtDNA microsatellite instability and the risk and prognosis needs to be further confirmed.

associated with the prognosis and recurrence of CRCs [72, 73]. The diagnostic value and prognostic evaluation value of mtMSI (mtDNA microsatellite instability) in CRCs remained undetermined, because the nMSI (nuclear microsatellite instability) had a unique value and significance in CRCs. We believed the mtMSI had a potential value on the risk and prognosis evaluation of colorectal cancer, although the researches in the field were relatively few.

The related literatures and research results of mtMSI in CRCs were showed in Table 4. Some studies suggested there was no association between the stromal mtMSI and stromal nMSI (nuclear microsatellite instability), and the stromal mtMSI was independent of stromal nuclear MSI (microsatellite instability) [66, 74]. A meta-analysis suggested mtMSI was higher in mCRC (metastatic colorectal cancer) patients with both of MSI and MSS in CRCs, but the prognosis was no correlation [51]. Some studies indicated mtMSI was higher in CRCs with Mn-SOD overexpression [67] and the (C)(n) repeat mtMSI was associated with tumor progression [66].

6. Discussion

The function of mitochondria was producing the adenosine triphosphate (ATP) via the oxidative phosphorylation system (OXPHOS) in normal physiology. It was generally acknowledged that the numbers of mitochondria showed an increase in high metabolism cells like heart muscle cells. Adequate amounts of energy were a necessary precondition for the uncontrolled rapid proliferation of cancer cells [50]. The number of mitochondria, such logic goes, was increased in the cancer tissues. However, the different researches got different or even contrary conclusions. The uncontrolled rapid proliferation, as the most important feature of cancer cells, was relative in vivo. Many factors including the pharmacological interventions, body's immune system, gene mutation, cancer cell heterogeneity, and nutritional deficiency restricted the proliferation of cancer cells. Thus, the mtDNA copy number as an independent biomarker to evaluate the risk and prognosis of colorectal cancer was inappropriate. But it might be more meaningful to act as the indicator of energy metabolism of cancers. Further studies should be focused on the association between the mtDNA copy number and energy metabolism, angiogenesis, and apoptotic cell proportion in vivo.

The expression of mitochondrial genes was in need of the assistance of the nuclear genes. The mitochondria retrograde cell signaling pathways illustrated that the mtDNA leaded to the changes of nDNA [75]. The number of aberrance mitochondria also affected the stress response and energy metabolism of the cancers. The variations of mutation sites and mutation frequency of mtDNA were found in the CRC tissues. There was a big disparity in the related literatures and research results because the study population had the different nationality, genders, ages, and living environment. We supported that the variations of mutation sites and mutation frequency of mtDNA could be used as auxiliary indicators to evaluate the risk and prognosis of CRC. But independent cohort studies with large sample size should be carried out to reduce the chance of confounding factors affecting investigation results.

The D-loop was the control region to regulate the replication and expression of the mitochondrial genome. More, the expression of the mitochondrial genome was controlled by nuclear DNA. The intertwined relationship between the mtDNA and nuclear DNA was unclear. There were large uncertainties about the association between the variations of mitochondrial genome and the risk and prognosis in CRCs. With the development of bioinformatics and gene sequencing technology, it might provide novel evidences for the mtDNA as the risk and prognosis factor in CRCs by decoding the molecular biological basis of tumorigenesis and progression and complex regulatory networks of interacting molecular components in the future. Besides, there were several interference factors of prognosis of CRCs including the treatment choices, patient's condition, the cancer stages, and biological behavior of cancers in the different researches and investigations. The large range, continuous, and dynamic surveillance of the changes of mtDNA was the further study directions to predict the value and role of the mtDNA.

Recent studies had shown that nuclear genome microsatellite instability was the significant predictor of prognosis CRCs [50, 72]. Much work remains to be done to make the

definitive relationship between the mtMSI and nMSI clear. But that did not mean the mtMSI could not act as an interesting predictor to evaluate the prognosis of CRCs. The researches in this field were relatively insufficient. In addition, mtDNA damage and repair system was essential for maintaining genome integrity and stability. Its relevant factors such as Tfam, POLG, and OGG1 may provide clues for the risk and prognosis evaluation of CRCs in the further study.

Moreover, there existed more empirical evidences to support the hypothesis that mtDNA and mitochondrial dysfunction could act as initiator in carcinogenesis. Intensive researches demonstrated that one or several mechanisms such as the mtDNA variation, mitochondrial dynamics [76], excessive quantity increases, mitochondrial enzyme defects [77], and mitochondrial retrograde signaling [78] could bring about global genomes changes that altered cell morphology and function, such as ATP production, calcium homeostasis, integration of metabolism, and regulation of apoptosis, and eventually leaded to tumor formation [79]. The mtDNA and mitochondrial dysfunction plays a vital role in the initiation and progression of malignancies and targeting the mtDNA might be a potential strategy for the development of selective anticancer therapy.

The review was to summarize current viewpoints of the controversial issues and described our understanding from the four aspects including mtDNA copy number, mitochondrial displacement loop, mtDNA variation, and mtDNA microsatellite instability. In conclusion, we believed that the mtDNA could serve as a potential biomarker for evaluating the risk and prognosis of colorectal cancer after conducting more in-depth studies. The summary of the mtDNA provided a meaningful reference and a valuable direction for the future studies.

Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

Authors' Contributions

All authors participated in the conception and design of the study. Han Shuwen and Pan Yuefen conceived the study and drafted the manuscript. Yang Xi made the tables. All authors read and approved the paper.

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References

[1] G. Gibson, "Cancer: directions for the drivers," *Nature*, vol. 512, no. 1, pp. 31–32, 2014.

[2] K. Lee and L. R. Ferguson, "MicroRNA biomarkers predicting risk, initiation and progression of colorectal cancer," *World Journal of Gastroenterology*, vol. 22, no. 33, pp. 7389–7401, 2016.

[3] X. Xie, B. Tang, Y.-F. Xiao et al., "Long non-coding RNAs in colorectal cancer," *Oncotarget*, vol. 7, no. 5, pp. 5226–5239, 2016.

[4] A. Alnabulsi and G. I. Murray, "Integrative analysis of the colorectal cancer proteome: potential clinical impact," *Expert Review of Proteomics*, vol. 13, no. 10, pp. 917–927, 2016.

[5] M. Yamamoto and S. Matsumoto, "Gut microbiota and colorectal cancer," *Genes and Environment*, vol. 38, no. 1, article 11, 2016.

[6] E. Silva Ramos, N. G. Larsson, and A. Mourier, "Bioenergetic roles of mitochondrial fusion," *Biochimica et Biophysica Acta*, vol. 1857, no. 8, pp. 1277–1283, 2016.

[7] F. Hosp, I. Lassowskat, V. Santoro et al., "Lysine acetylation in mitochondria: from inventory to function," *Mitochondrion*, vol. 33, pp. 58–71, 2017.

[8] S. Shiratsuki, T. Hara, Y. Munakata, K. Shirasuna, T. Kuwayama, and H. Iwata, "Low oxygen level increases proliferation and metabolic changes in bovine granulosa cells," *Molecular and Cellular Endocrinology*, vol. 437, pp. 75–85, 2016.

[9] X. K. Xing, M. H. Li, Z. W. Guo, and C. S. Xu, "Expression profiles of genes associated with mitochondria-mediated apoptosis and their roles in liver regeneration," *Genetics and Molecular Research*, vol. 15, no. 3, 2016.

[10] A. Latorre-Pellicer, R. Moreno-Loshuertos, A. V. Lechuga-Vieco et al., "Mitochondrial and nuclear DNA matching shapes metabolism and healthy ageing," *Nature*, vol. 535, no. 7613, pp. 561–565, 2016.

[11] M. Kubota, Y. B. Shui, M. Liu et al., "Mitochondrial oxygen metabolism in primary human lens epithelial cells: association with age, diabetes and glaucoma," *Free Radical Biology and Medicine*, vol. 97, pp. 513–519, 2016.

[12] C. Ridler, "Obesity: inheritance via mitochondria," *Nature Reviews Endocrinology*, vol. 12, no. 9, article 497, 2016.

[13] M. Guzmán-Fulgencio, J. L. Jiménez, M. García-Álvarez et al., "Mitochondrial haplogroups are associated with clinical pattern of AIDS progression in HIV-infected patients," *Journal of Acquired Immune Deficiency Syndromes*, vol. 63, no. 2, pp. 178–183, 2013.

[14] R. M. Andrews, I. Kubacka, P. F. Chinnery, R. N. Lightowlers, D. M. Turnbull, and N. Howell, "Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA," *Nature Genetics*, vol. 23, no. 2, p. 147, 1999.

[15] P. F. Chinnery and G. Hudson, "Mitochondrial genetics," *British Medical Bulletin*, vol. 106, no. 1, pp. 135–159, 2013.

[16] B. Korzeniewski, M. Malgat, T. Letellier, and J.-P. Mazat, "Effect of 'binary mitochondrial heteroplasmy' on respiration and ATP synthesis: Implications for mitochondrial diseases," *Biochemical Journal*, vol. 357, no. 3, pp. 835–842, 2001.

[17] R. Rossignol, M. Malgat, J.-P. Mazat, and T. Letellier, "Threshold effect and tissue specificity. Implication for mitochondrial cytopathies," *Journal of Biological Chemistry*, vol. 274, no. 47, pp. 33426–33432, 1999.

[18] D. C. Wallace, "Mitochondrial diseases in man and mouse," *Science*, vol. 283, no. 5407, pp. 1482–1488, 1999.

[19] M. Wachsmuth, A. Hübner, M. Li, B. Madea, and M. Stoneking, "Age-related and heteroplasmy-related variation in human mtDNA copy number," *PLoS Genetics*, vol. 12, no. 3, Article ID e1005939, 2016.

[20] F. H. M. van Osch, A. M. Voets, L. J. Schouten et al., "Mitochondrial DNA copy number in colorectal cancer: between tissue comparisons, clinicopathological characteristics and survival," *Carcinogenesis*, vol. 36, no. 12, pp. 1502–1510, 2015.

[21] Y. Wang, V. W. S. Liu, W. C. Xue, A. N. Y. Cheung, and H. Y. S. Ngan, "Association of decreased mitochondrial DNA content with ovarian cancer progression," *British Journal of Cancer*, vol. 95, no. 8, pp. 1087–1091, 2006.

[22] Y. Wang, S. He, X. Zhu, W. Qiao, and J. Zhang, "High copy number of mitochondrial DNA predicts poor prognosis in patients with advanced stage colon cancer," *The International Journal of Biological Markers*, vol. 31, no. 4, pp. e382–e388, 2016.

[23] A. M. S. Haja Mohideen, E. Dicks, P. Parfrey, R. Green, and S. Savas, "Mitochondrial DNA polymorphisms, its copy number change and outcome in colorectal cancer," *BMC Research Notes*, vol. 8, article 272, 2015.

[24] S. Wen, J. Gao, L. Zhang, H. Zhou, D. Fang, and S. Feng, "p53 increase mitochondrial copy number via up-regulation of mitochondrial transcription factor A in colorectal cancer," *Oncotarget*, vol. 7, no. 46, pp. 75981–75995, 2016.

[25] B. Thyagarajan, W. Guan, V. Fedirko et al., "No association between mitochondrial DNA copy number and colorectal adenomas," *Molecular Carcinogenesis*, vol. 55, no. 8, pp. 1290–1296, 2016.

[26] B. Thyagarajan, R. Wang, H. Barcelo, W.-P. Koh, and J.-M. Yuan, "Mitochondrial copy number is associated with colorectal cancer risk," *Cancer Epidemiology Biomarkers and Prevention*, vol. 21, no. 9, pp. 1574–1581, 2012.

[27] F. Qu, Y. Chen, X. Wang et al., "Leukocyte mitochondrial DNA content: a novel biomarker associated with prognosis and therapeutic outcome in colorectal cancer," *Carcinogenesis*, vol. 36, no. 5, pp. 543–552, 2015.

[28] B. Huang, Y.-T. Gao, X.-O. Shu et al., "Association of leukocyte mitochondrial DNA copy number with colorectal cancer risk: results from the Shanghai Women's Health Study," *Cancer Epidemiology Biomarkers and Prevention*, vol. 23, no. 11, pp. 2357–2365, 2014.

[29] H. Sultana, D. W. Seo, M. S. Bhuiyan et al., "Genetic diversity and phylogenetic analysis of South-East Asian duck populations based on the mtDNA D-loop sequences," *Asian-Australasian Journal of Animal Sciences*, vol. 29, no. 12, pp. 1688–1695, 2016.

[30] J. Zhang, Z. Guo, Y. Bai et al., "Identification of sequence polymorphisms in the displacement loop region of mitochondrial DNA as a risk factor for renal cell carcinoma," *Biomedical Reports*, vol. 1, pp. 563–566, 2013.

[31] J.-W. Taanman, "The mitochondrial genome: structure, transcription, translation and replication," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, vol. 1410, no. 2, pp. 103–123, 1999.

[32] S. Feng, L. Xiong, Z. Ji, W. Cheng, and H. Yang, "Correlation between increased ND2 expression and demethylated displacement loop of mtDNA in colorectal cancer," *Molecular Medicine Reports*, vol. 6, no. 1, pp. 125–130, 2012.

[33] J. Gao, S. Wen, H. Zhou, and S. Feng, "De-methylation of displacement loop of mitochondrial DNA is associated with increased mitochondrial copy number and nicotinamide adenine dinucleotide subunit 2 expression in colorectal cancer," *Molecular Medicine Reports*, vol. 12, no. 5, pp. 7033–7038, 2015.

[34] S. Govatati, B. Saradamma, S. Malempati et al., "Association of mitochondrial displacement loop polymorphisms with risk of colorectal cancer in south Indian population," *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis*, 2016.

[35] Y. Bai, Z. Guo, J. Xu et al., "Single nucleotide polymorphisms in the D-loop region of mitochondrial DNA is associated with renal cell carcinoma outcome," *Mitochondrial DNA*, vol. 26, no. 2, pp. 224–226, 2015.

[36] A. Legras, A. Lièvre, C. Bonaiti-Pellié et al., "Mitochondrial D310 mutations in colorectal adenomas: an early but not causative genetic event during colorectal carcinogenesis," *International Journal of Cancer*, vol. 122, no. 10, pp. 2242–2248, 2008.

[37] A. M. Kassem, N. El-Guendy, M. Tantawy, H. Abdelhady, A. El-Ghor, and A. H. Abdel Wahab, "Mutational hotspots in the mitochondrial D-loop region of cancerous and precancerous colorectal lesions in egyptian patients," *DNA and Cell Biology*, vol. 30, no. 11, pp. 899–906, 2011.

[38] M. Akouchekian, M. Houshmand, S. Hemati, M. Ansaripour, and M. Shafa, "High rate of mutation in mitochondrial DNA displacement loop region in human colorectal cancer," *Diseases of the Colon and Rectum*, vol. 52, no. 3, pp. 526–530, 2009.

[39] S. C. Chang, P. C. Lin, S. H. Yang, H. S. Wang, W. Y. Liang, and J. K. Lin, "Mitochondrial D-loop mutation is a common event in colorectal cancers with p53 mutations," *International Journal of Colorectal Disease*, vol. 24, no. 6, pp. 623–628, 2009.

[40] A. Lièvre, C. Chapusot, A.-M. Bouvier et al., "Clinical value of mitochondrial mutations in colorectal cancer," *Journal of Clinical Oncology*, vol. 23, no. 15, pp. 3517–3525, 2005.

[41] L. F. de Araujo, A. S. Fonseca, B. R. Muys et al., "Mitochondrial genome instability in colorectal adenoma and adenocarcinoma," *Tumor Biology*, vol. 36, no. 11, pp. 8869–8879, 2015.

[42] H. Cui, P. Huang, Z. Wang et al., "Association of decreased mitochondrial DNA content with the progression of colorectal cancer," *BMC Cancer*, vol. 13, article 110, 2013.

[43] S. Feng, L. Xiong, Z. Ji, W. Cheng, and H. Yang, "Correlation between increased copy number of mitochondrial DNA and clinicopathological stage in colorectal cancer," *Oncology Letters*, vol. 2, no. 5, pp. 899–903, 2011.

[44] S. W. Lim, H. R. Kim, H. Y. Kim et al., "High-frequency minisatellite instability of the mitochondrial genome in colorectal cancer tissue associated with clinicopathological values," *International Journal of Cancer*, vol. 131, no. 6, pp. 1332–1341, 2012.

[45] H.-C. Lee, P.-H. Yin, J.-C. Lin et al., "Mitochondrial genome instability and mtDNA depletion in human cancers," *Annals of the New York Academy of Sciences*, vol. 1042, pp. 109–122, 2005.

[46] W. R. R. Geurts-Giele, G. H. G. K. Gathier, P. N. Atmodimedjo, H. J. Dubbink, and W. N. M. Dinjens, "Mitochondrial D310 mutation as clonal marker for solid tumors," *Virchows Archiv*, vol. 467, no. 5, pp. 595–602, 2015.

[47] C. Wang, S. Zhao, Y. Du, and Z. Guo, "Single nucleotide polymorphisms in the D-loop region of mitochondrial DNA is associated with colorectal cancer outcome," *Mitochondrial DNA Part A: DNA Mapping, Sequencing, and Analysis*, vol. 27, no. 6, pp. 4361–4363, 2016.

[48] E. Errichiello, A. Balsamo, M. Cerni, and T. Venesio, "Mitochondrial variants in MT-CO2 and D-loop instability are involved in MUTYH-associated polyposis," *Journal of Molecular Medicine*, vol. 93, no. 11, pp. 1271–1281, 2015.

[49] K. Hibi, H. Nakayama, T. Yamazaki et al., "Detection of mitochondrial DNA alterations in primary tumors and corresponding serum of colorectal cancer patients," *International Journal of Cancer*, vol. 94, no. 3, pp. 429–431, 2001.

[50] Z. Gatalica, S. Vranic, J. Xiu, J. Swensen, and S. Reddy, "High microsatellite instability (MSI-H) colorectal carcinoma: a brief review of predictive biomarkers in the era of personalized medicine," *Familial Cancer*, vol. 15, no. 3, pp. 405–412, 2016.

[51] S. Venderbosch, S. van Vliet, M. H. C. Craenmehr et al., "Mitochondrial microsatellite instability in patients with metastatic colorectal cancer," *Virchows Archiv*, vol. 466, no. 5, pp. 495–502, 2015.

[52] K. Linkowska, A. Jawien, A. Marszałek, K. Skonieczna, and T. Grzybowski, "Searching for association of the CAG repeat polymorphism in the mitochondrial DNA polymerase gamma gene (POLG) with colorectal cancer," *Acta Biochimica Polonica*, vol. 62, no. 3, pp. 625–627, 2015.

[53] F. Mohammed, A. R. Rezaee khorasany, E. Mosaieby, and M. Houshmand, "Mitochondrial A12308G alteration in tRNA-(Leu(CUN)) in colorectal cancer samples," *Diagnostic Pathology*, vol. 10, no. 1, article 115, 2015.

[54] T. Miwata, T. Hiyama, D. T. R. Quach et al., "Differences in K-ras and mitochondrial DNA mutations and microsatellite instability between colorectal cancers of Vietnamese and Japanese patients," *BMC Gastroenterology*, vol. 14, article 203, 2014.

[55] J. Dimberg, T. T. Hong, M. Skarstedt, S. Löfgren, N. Zar, and A. Matussek, "Novel and differential accumulation of mitochondrial DNA deletions in Swedish and Vietnamese patients with colorectal cancer," *Anticancer Research*, vol. 34, no. 1, pp. 147–152, 2014.

[56] N. G. Ericson, M. Kulawiec, M. Vermulst et al., "Decreased mitochondrial DNA mutagenesis in human colorectal cancer," *PLoS Genetics*, vol. 8, no. 6, Article ID e1002689, 2012.

[57] T. C. Larman, S. R. DePalma, A. G. Hadjipanayis et al., "Spectrum of somatic mitochondrial mutations in five cancers," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 35, pp. 14087–14091, 2012.

[58] C.-Y. Wang, H. Li, X.-D. Hao et al., "Uncovering the profile of somatic mtDNA mutations in Chinese Colorectal cancer patients," *PLoS ONE*, vol. 6, no. 6, Article ID e21613, 2011.

[59] T. Shimomura, T. Hiyama, S. Oka et al., "Frequent somatic mutations of mitochondrial DNA in traditional serrated adenomas but not in sessile serrated adenomas of the colorectum," *Journal of Gastroenterology and Hepatology*, vol. 26, no. 10, pp. 1565–1569, 2011.

[60] M. Akouchekian, M. Houshmand, M. H. H. Akbari, B. Kamalidehghand, and M. Dehghane, "Analysis of mitochondrial ND1 gene in human colorectal cancer," *Journal of Research in Medical Sciences*, vol. 16, no. 1, pp. 50–55, 2011.

[61] Y. Yusnita, M. D. Norsiah, and A. J. A Rahman, "Mutations in mitochondrial NADH dehydrogenase subunit 1 (mtND1) gene in colorectal carcinoma," *Malaysian Journal of Pathology*, vol. 32, no. 2, pp. 103–110, 2010.

[62] T. Chen, J. He, L. Shen et al., "The mitochondrial DNA 4,977-bp deletion and its implication in copy number alteration in colorectal cancer," *BMC Medical Genetics*, vol. 12, article 8, 2011.

[63] M. Pinheiro, I. Veiga, C. Pinto et al., "Mitochondrial genome alterations in rectal and sigmoid carcinomas," *Cancer Letters*, vol. 280, no. 1, pp. 38–43, 2009.

[64] E. Webb, P. Broderick, I. Chandler et al., "Comprehensive analysis of common mitochondrial DNA variants and colorectal cancer risk," *British Journal of Cancer*, vol. 99, no. 12, pp. 2088–2093, 2008.

[65] J.-H. Lee, I. Hwang, Y.-N. Kang, I.-J. Choi, and D.-K. Kim, "Genetic characteristics of mitochondrial DNA was associated with colorectal carcinogenesis and its prognosis," *PLoS ONE*, vol. 10, no. 3, Article ID e0118612, 2015.

[66] H. S. Kim, H. S. Lim, S. H. Lee et al., "Mitochondrial microsatellite instability of colorectal cancer stroma," *International Journal of Cancer*, vol. 119, no. 11, pp. 2607–2611, 2006.

[67] S. Govatati, S. Malempati, B. Saradamma et al., "Manganese-superoxide dismutase (Mn-SOD) overexpression is a common event in colorectal cancers with mitochondrial microsatellite instability," *Tumor Biology*, vol. 37, no. 8, pp. 10357–10364, 2016.

[68] H. Takeuchi, A. Fujimoto, and D. S. B. Hoon, "Detection of mitochondrial DNA alterations in plasma of malignant melanoma patients," *Annals of the New York Academy of Sciences*, vol. 1022, pp. 50–54, 2004.

[69] G. Adams Jr., S. Mehrabi, Y. Vatcharapijarn et al., "Frequencies of mtDNA mutations in primary tissues of colorectal adenopolyps," *Frontiers in Bioscience (Elite Edition)*, vol. 5, no. 3, pp. 809–813, 2013.

[70] N. Chihara, T. Amo, A. Tokunaga et al., "Mitochondrial DNA alterations in colorectal cancer cell lines," *Journal of Nippon Medical School*, vol. 78, no. 1, pp. 13–21, 2011.

[71] H. Ashktorab, S. Ahuja, L. Kannan et al., "A meta-analysis of MSI frequency and race in colorectal cancer," *Oncotarget*, vol. 7, no. 23, pp. 34546–34557, 2016.

[72] M. Srdjan, A. Jadranka, D. Ivan et al., "Microsatellite instability & survival in patients with stage II/III colorectal carcinoma," *The Indian Journal of Medical Research*, vol. 143, pp. S104–S111, 2016.

[73] E. N. Mojarad, S. M. H. Kashfi, H. Mirtalebi et al., "Low level of microsatellite instability correlates with poor clinical prognosis in stage II colorectal cancer patients," *Journal of Oncology*, vol. 2016, Article ID 2196703, 9 pages, 2016.

[74] G. Guleng, T. Løvig, G. I. Meling, S. N. Andersen, and T. O. Rognum, "Mitochondrial microsatellite instability in colorectal carcinomas—frequency and association with nuclear microsatellite instability," *Cancer Letters*, vol. 219, no. 1, pp. 97–103, 2005.

[75] F. M. da Cunha, N. Q. Torelli, and A. J. Kowaltowski, "Mitochondrial retrograde signaling: triggers, pathways, and outcomes," *Oxidative Medicine and Cellular Longevity*, vol. 2015, Article ID 482582, 10 pages, 2015.

[76] S. Srinivasan, M. Guha, A. Kashina, and N. G. Avadhani, "Mitochondrial dysfunction and mitochondrial dynamics—the cancer connection," *Biochimica et Biophysica Acta (BBA)—Bioenergetics*, 2017.

[77] M. Miyo, M. Konno, H. Colvin et al., "The importance of mitochondrial folate enzymes in human colorectal cancer," *Oncology Reports*, vol. 37, no. 1, pp. 417–425, 2017.

[78] L. Formentini, M. Sánchez-Aragó, L. Sánchez-Cenizo, and J. M. C. Cuezva, "The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde prosurvival and proliferative response," *Molecular Cell*, vol. 45, no. 6, pp. 731–742, 2012.

[79] C. C. Hsu, L. M. Tseng, and H. C. Lee, "Role of mitochondrial dysfunction in cancer progression," *Experimental Biology and Medicine*, vol. 241, no. 12, pp. 1281–1295, 2016.