Molecular Genetics and Cytogenetics in Cancer

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Editorial Board

Contents

Editorial

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This special issue of Genetics Research International focuses on *molecular genetics and cytogenetics in cancer*. The hunt for informative disease-related genetic biomarkers may become so valuable for the differential diagnosis, prognosis, and disease monitoring of cancer patients that numerous researchers are committed to this challenge. Indeed, a colossal effort of current molecular medicine to progress in the understanding of the hallmarks of cancer is focusing on the field of biomarker discovery through genetic and cytogenetic profiling. Ambitious initiatives such as the Cancer Genome Atlas (http://cancergenome.nih.gov/) or the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/cgap.html) from the US National Institutes of Health (NIH) are devoted to provide collaborative platforms to better comprehend and fight the malignant signature of cancer.

The fast development of high-throughput technologies is bringing to biomedical research more affordable and high-resolution ways to explore whole genomes, chromosomes, genes, proteins, RNA, and metabolites. In consequence, numerous genomic datasets accumulate in international repositories, such as GEO from the US National Center for Biotechnology Information (NCBI), and around 500,000 clonal chromosomal abnormalities belonging to more than 60,000 human neoplasms are described in the latest catalogue of chromosome aberrations in cancer. However, translational application of biomarker discovery still has to face its ultimate challenge: incorporation into routine clinical practice.

We are under the pressure to combine the multidisciplinary efforts of basic, clinical, and computational research fields to identify meaningful genetic profiles that can be used as powerful tools for diagnosis, prognosis, and treatment. Every patient who suffers from cancer hopes that our research will deliver alternatives. For scientists, the identification of the specific genetic abnormalities responsible for tumor diversity and heterogeneity is crucial to shed light on the molecular mechanisms of cancer and the identification of druggable targets and functional pathways. For clinicians, the appropriate molecular categorization of patients would allow to enroll them in tailored—more adequate and less toxic—treatments, improving healthcare and optimizing medical costs.

Ultimately, any effort to (i) frame the important unsolved problems, (ii) to review what is known about the molecular pathways involved in the pathogenesis of distinct types of cancer, (iii) to catalog recurrent genetic alterations that could serve as biomarkers, (iv) to suggest standardization of routine protocols of diagnosis and molecular detection, and (v) to rank available or potential drugs and their targets, is decisive to help molecular medicine in its challenge against cancer. This is the scope of this special issue where the biographies of some of the most aggressive hematological and solid tumors are discussed. The journal Genetics Research International is serving here as a forum for discussion and review of major genetic risks involved in breast cancer metastasis to the brain as well as in the evolution of myxoid soft-tissue sarcomas and parathyroid tumors. The importance of applying genetic protocols in the risk-based stratification of multiple myeloma is also reviewed here, and new evidences supporting genetic aberrations in a rare variant of T-cell lymphomas known as Sézary syndrome are presented. Finally,

myeloid malignancies are revealed as a paradigm to discuss the role of dicentric chromosomes as instigators of genomic instability and suggest a model for their implication in the formation of unbalanced translocations in malignancy.

Breaking the barriers of cancer is a difficult task that is continuously challenging the research community.

We, the editors of this special issue, are hopeful that the ideas presented here can inspire the critical thinking of the specialized readers and contribute to the common efforts to better understand and fight the multiple faces of cancer.

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Review Article

Molecular Aspects of Breast Cancer Metastasis to the Brain

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Our knowledge of the biology underlying the development of brain metastases (BM) from breast cancer has improved over the last decade due to large clinical epidemiological studies, animal models of metastasis, and the use of high-resolution gene expression profiling technologies. However, there are still major gaps in our understanding of the mechanisms utilized by breast cancer cells to colonize the brain microenvironment, thus our arsenal of therapies remains relatively nonspecific, and the prognosis for breast cancer patients with BM remains poor. Additional insights into these mechanisms are necessary to facilitate the development of new preventive and curative therapeutic regimens to block this fatal disease. This paper aims to provide a general overview for the readers of what has been achieved in this field of research and its translation into clinical practice to date and to highlight exciting new areas of research that promise to inform the development of new targeted therapies for BM.

1. An Overview of Metastasis

Metastasis, or metastatic disease, is the spread of cancer cells from one organ to a distant site via the blood or lymph. In the Nineteenth Century, Paget asked whether the distribution of metastases in different organs was simply a matter of chance. He studied autopsy records of women with breast cancer, revealing a nonrandom pattern of metastatic colonization. He proposed a hypothesis that tumour cells (the "seed") could have specific affinity for the microenvironments of certain organs (the "soil") [1]. This intriguing phenomenon is called organotropism, and Paget's hypothesis has now been repeatedly substantiated, with growing recognition of the importance of tumour cell interactions with the stromal microenvironment in supporting the establishment of metastases (see below). For instance, analysis of large autopsy series has showed that lung, breast, melanoma, renal, and colon cancers are the most common primary tumours to metastasize to the brain [2, 3]. There is a theory that the primary tumour could contribute to priming "premetastatic niches" prior to the establishment of micromestastases, thereby influencing organ tropism. Kaplan, Lyden, and colleagues' analysis

of mouse models of lung metastasis implicated certain chemokines in this process, as well as mobilisation of haematopoietic precursor cells to pre-metastatic sites [4], however the mechanisms underlying creation of pre-metastatic niches are not fully understood [5].

The metastatic process is very inefficient. In order to accomplish distant metastasis, tumour cells must first detach and/or escape from the primary site, then survive as circulating tumour cells (CTCs) in the absence of the microenvironment cues with which they were conceived. Most CTCs are cleared from initial trapping sites within a few days. Those that survive, and succeed in extravasating, engraft at a distant site forming a micrometastasis, then may proliferate to form a clinically significant lesion after a fairly unpredictable period of latency (dormancy) spent meeting requirements for cell division in the new microenvironment [6, 7]. The poorly understood "dormancy" phenomenon poses a major challenge in metastasis management. Despite being attractive drug targets (at least conceptually), CTCs and micrometastases are undetectable with current hematologic and imaging technologies and are thought to be insensitive to chemotherapeutics that target rapidly dividing cells.

It is now becoming clear that only a very small proportion of primary tumour cells are capable of forming clinically significant metastatic lesion [8]. Because these cells share critical features with normal stem cells (namely, multi-lineage potential in reseeding a secondary tumour and self-renewal), they have been called cancer stem cells (CSCs). It is hypothesized that a complex combination of tumour cell intrinsic and extrinsic processes eventually culminates in the activation of dormant CSC proliferation at distant sites [8]. Current evidence suggests that this includes continuing tumour cell genetic evolution, adaptation, and selection of those cells with signaling programs that best engage and exploit the new microenvironment [9, 10]. Observations on the temporal courses of metastasis, from different primary tumour types, provide some insight into the biology underlying these processes. For example, subsets of breast and lung carcinomas show similar overall organ tropism (brain, bone, lung, and liver), but strikingly different progression times, with distant relapse detected early in lung cancer (macrometastases established within months of diagnosis), and relatively late in breast cancer progression (after years to decades of remission). This suggests early acquisition of metastasis-enabling genetic alterations in lung cancer, in contrast to a longer latency period as CTCs and/or micrometastases in breast cancer.

At least three categories of metastasis genes have been proposed to facilitate the multistep metastatic cascade (reviewed in [11]): (1) "initiation" genes that facilitate detachment (e.g., *CDH2* (encodes N-Cadherin) and *TWIST)*, extracellular matrix degradation (e.g., *MMP*s) or angiogenesis (e.g., *VEGF*); (2) "progression" genes (e.g., *PTGS2* (encodes COX-2) and *MMP-1)* that regulate extravasation of circulating tumour cells and are involved in metastatic colonisation; (3) "virulence" genes (e.g., *IL6* and *TNFα*), which promote survival in circulation, and/or provide a proliferative advantage in the distant microenvironment. Apart from these metastasis-promoting genes, there is a well-distinguished class of metastasis "suppressor" genes that represses tumour cell dissemination without any effect on primary tumour growth, including *KAI-1*, *BRSM1*, and *NME1* [11]. These findings are mainly derived from studies using animal models. Historically, it has been difficult to understand the genetic alterations underlying metastasis by direct analysis of human tissue. Transcriptomic profiling of matched pairs of primary tumours and their metastases has demonstrated a high degree of similarity with minor differential gene expression [12–14]. However, the more recent application of next-generation whole genome sequencing (WGS) technologies is revealing subtle, but significant distinctions between metastases and their primary tumours of origin [15, 16] that were previously undetectable.

2. What Is the Impact of Brain Metastasis in the Natural History of Breast Cancer?

The prevalence of BM during the course of breast cancer disease has been reported to range from 10%–16%, reaching 30% when autopsy diagnoses of BM are included [17, 18]. Several factors have been reported to be associated with a higher risk of developing BM: patients less than fifty years old, four or more axillary lymph nodes involved with metastatic disease, basal phenotype, and high tumour grade [19–22]. Current therapeutic strategies for BM include whole brain radiation therapy (WBRT; the treatment mainstay since the 1950s), stereotactic radiosurgery, or surgery combined with radiotherapy. The median survival in untreated breast cancer patients with symptomatic BM is less than one month, 6–8 weeks in patients treated with steroids alone and 3–6 months when treated with WBRT [3, 23].

Patients with HER2-positive breast tumours are now also regarded to be at high risk for developing BM. Improved control of systemic disease with the anti-HER2 monoclonal antibody combined with poor BBB penetration have been regarded as culprits of this increased metastatic trend [24]. BM treatment strategies are currently informed by the histopathology of the primary tumour, and BM are rarely biopsied. Whilst many features are shared, it is becoming clear that metastases are distinct in their genetic landscape and expression of critical disease markers [12, 14–16]. Hence, future therapeutic development is likely to be based on features of the metastases themselves.

3. Does the Incidence of BM Correlate with Breast Cancer Molecular Subtypes?

Breast cancer is a heterogeneous disease with respect to molecular features perhaps best exemplified by the molecular subgroups identified by gene expression profiling including basal-like, luminal A (hormone receptor positive), luminal B, and HER2 amplified/over-expressed (HER2+) subtypes. These molecular subtypes are associated with different outcomes. It is generally accepted that the basal-like, luminal B and HER2+ subtypes are associated with particularly poor prognosis compared with the hormone receptor-positive, luminal A subtype [25, 26] (Figure 1). Basal-like tumours are generally high grade, have central areas of necrosis, invasive pushing borders and are characterized by the expression of markers including high-molecular weight cytokeratins (e.g., CK14 and CK5/6), p53 and the myoepithelial markers Smooth Muscle Actin (SMA) and p63 [27, 28].

We, and other groups, have reported a greater propensity of primary breast cancer with a basal-like immunophenotype to metastasize to the brain [20–22]. Moreover, patients with germline *BRCA1* mutations who develop breast cancer have a higher incidence of BM compared to germline *BRCA2* carriers and non-*BRCA1/2* patients [29]. These tumours have morphological similarities with those from the sporadic basal-like group [27, 28]. Patients with HER2+ tumours have also been shown to have an increased incidence of BM [30]. These metastatic patterns were further validated by a large study analysing, 3,726 cases, with a median follow-up of 14.8 years [31]. This work confirmed a higher rate of BM in HER2+-enriched and basal-like, compared to luminal A tumours. The cumulative incidence for BM in basal-like and HER2+ tumours were highest in the first five years after diagnosis, plateauing thereafter.
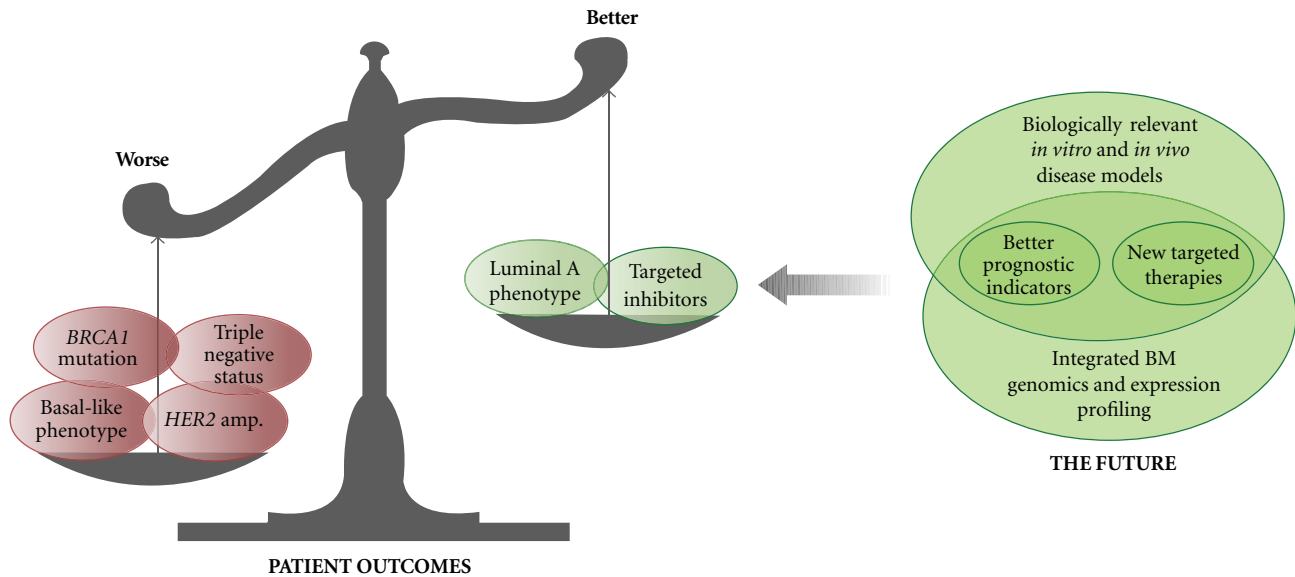
FIGURE 1: Summary of the major factors that increase or decrease risk of developing brain metastases in breast cancer and exciting new areas of research that promise to deliver the knowledge required for new targeted therapies and better prognostication.

4. Current Understanding of BM from Breast Cancer Mechanisms

Gene expression profiling of breast tumours coupled with outcome data, functional analyses on cell lines, and *in vivo* animal models have shed light on our understanding of the colonization of the brain parenchyma by breast cancer cells [10, 11, 16, 42–44] (Figure 1).

Regarding the brain microenvironment, there are two main cell types in the neural tissue: neurons and glial cells, including microglia, astroglia, and oligodendroglia. There is a body of evidence suggesting that metastatic tumour cell interactions with the brain microenvironment facilitate the colonization process [43]. Interactions between breast cancer cells and pericytes and/or astrocytes might be responsible for alterations in the BBB and thus, development of BM from breast cancer. For example, Mendes et al. [45] reported that astrocyte-induced factors activated the ERK1/2 signalling pathway in rat mammary adenocarcinoma ENU1564 cells and thus enhanced the invasive features of these cells through increased expression of MMP-2. Furthermore, transfection of ENU1564 cells with TIMP-2, a natural inhibitor of MMP-2, reduced the *in vitro* invasive characteristics of these cells. BM was not observed in animals inoculated with ENU1564-TIMP-2, which implies a cardinal role for MMP-2 in BMBC [45]. In addition, reactive glia under coculture with breast cancer cell line MDA-MB-231 was shown to enhance growth of this same cell line [46]. Furthermore, the 435-Br1 cell line, which was derived from BM in a nude mouse, showed increased adhesion to astrocytes and enhanced growth *in vitro* in the presence of an astrocyte-conditioned media when compared to parental MDA-MB-435 breast cancer cells and the lung metastasis-derived variant 435-Lung2 [47]. Table 1 summarizes BM-associated genes identified in integrated studies that used combinations of *in vitro* cell culture-based functional assays, *in vivo* mouse xenograft models and the analysis of human clinical samples.

Palmieri et al. demonstrated that when human MDA-MB-231-BR cells, the brain metastatic derivative of the MDA-MB-231 cell line, were transfected with HER2, the HER2-overexpressing clones showed a threefold increase in the number of large BM compared with control MDA-MB-231-BR cells [39]. These findings are consistent with epidemiological studies showing increased incidence of BM in HER2+ breast cancer patients [30]. The same group showed *in vitro* that Lapatinib inhibited the phosphorylation of EGFR, HER2, and downstream signalling proteins leading to reduced proliferation and migration in 231-BR. This *in vitro* data was also replicated in a mouse model showing Lapatinib inhibited the growth of brain macrometastases seeded from EGFR-overexpressing 231-BR cells [48].

Another example of an integrated approach to understanding BM from breast cancer was published by Bos et al. In this study, cyclooxygenase, the epidermal growth factor receptor ligand HBEGF and α2,6-sialyltransferase acted as mediators of cancer cell passage through the BBB. Again, these findings were derived from *in vitro* functional analyses and animal models, with translation in clinical samples where meta-analysis of gene expression profiling of patients who had brain metastases and survival data were combined. The presence of this signature was related to shorter brain-metastasis free survival [34].

Several studies focusing on the functional significance of single genes have also contributed to our current understanding of BM development. In an effort to develop an experimental model of BM from breast cancer, Kim et al. used internal carotid artery injection of breast cancer cells into nude mice, which resulted in formation of different brain metastatic variant cell lines. These variants expressed higher levels of vascular endothelial growth factor (*VEGF*)

TABLE 1: Genes implicated in the development of brain metastases from breast cancer.

Gene abbrev	Gene name	BM expression status	Gene product functions	Reference
KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	↑	Voltage gated ion channel involved in neuronal excitability	[32]
MNT	MAX binding protein	↓	Myc antagonist	[33]
TERC	Telomerase RNA component	↑	A template for telomere repeat	[33]
CTSB	Cathepsin B	↑	Lysosomal cysteine proteinase	[33]
PTGS2	Prostaglandin-endoperoxide synthase 2	↑	Prostaglandin biosynthesis	[34]
HBEGF	Heparin-binding EGF-like growth factor	↑	EGFR signalling	[34]
ST6GALNAC5	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5	↑	Sialyltransferase that modifies proteins and ceramides	[34]
CXCR4	Chemokine (C-X-C motif) receptor 4	↑	Receptor for stromal cell-derived factor-1	[35]
MMP2	Matrix metallopeptidase 2	↑	Degradation of extracellular matrix	[36]
MMP9	Matrix metallopeptidase 9	↑	Degradation of extracellular matrix	[36]
ROBO1	Roundabout	↑	Axon guidance and neuronal precursor cell migration	[37]
ERBB3	Y-erb-b2 erythroblastic leukemia viral oncogene homolog 3	↑	Cell proliferation and differentiation	[12]
CAV1	Caveolin 1, caveolae protein	↓	Structural component of the caveolae plasma membranes	[38]
ERBB2	v-erb b2 erythroblastic leukemia viral oncogene homolog 2	↑	Cell proliferation	[39]
HK2	Hexokinase 2	↑	Glycolysis	[34]
HPSE	Heparanase	↑	Remodeling of the extracellular matrix	[40]
STAT3	Signal transducer and activator of transcription 3	↑	Cell growth and apoptosis, inflammation, invasion, and metastasis	[39]
VEGF	Vascular endothelial growth factor	↑	Stimulates angiogenesis and vasculogenesis	[41]
IL-8	Interleukin 8	↑	CXC chemokine involved in neutrophil recruitment	[41]

↑ overexpressed; ↓ downregulated.

and IL-8 than the nonbrain metastatic clones, suggesting a possible role for VEGF in BM from breast cancer [40]. Consistent with this, suppression of BM and induction of apoptosis were observed following treatment of the mice with the VEGF-receptor tyrosine kinase inhibitor, PTK787/Z 222584 [40]. A second study by Palmieri et al. analyzed gene expression profiles of laser-captured epithelial cells from BM of breast cancer patients compared with unmatched primary breast tumors. The results showed that hexokinase 2 (HK2), a critical enzyme in glucose metabolism, is increased in BM [49]. Increased expression of HK2 was observed in the 231-BR brain metastatic breast cancer cell line [49]. A third example is the study by Chiu et al., which showed augmented expression of the activated form of signal transducer and activator of transcription 3 (STAT3) coupled with downregulation of Caveolin-1 (the structural component of caveolae involved in membrane trafficking and cell signaling) in BM compared with primary breast tumours. Furthermore, they showed that ectopic expression of Caveolin-1 or knockdown of STAT3 reduces the invasive features of breast cancer cells in vitro and brain colonization in vivo [38].

Our group has combined gene expression array profiling, targeted somatic mutation analysis, and immunohistochemical profiling of BM from breast cancer and integrated the data to demonstrate activation of signaling pathways associated with the HER receptor family in BM compared to their matched primary breast tumours. Critically, the data showed an increase in HER3 expression in breast cancer cells isolated from BM compared to matched primary tumours [12]. Neuregulin 1, the ligand for this receptor, is abundantly expressed in the brain [50] and is activated by a variety of stimuli, including hypoxia [51]. Consistent with this, we observed increased expression of hypoxia-inducible Factor 1α (HIF-1α) in the BM, likely reflecting the local hypoxic environment. Increased HER3 expression has also been reported in BM from lung cancer [52]. These two clinical snapshots could reflect environmental selection and therefore adaptation of metastatic cells to the brain microenvironment, exploiting the Neuregulin-HER3 axis in order to succeed.

While there is ample evidence that miRNAs have determinant roles in tumour cell dissemination, the possible roles of these "micromanagers of metastasis" in BM from breast

cancer are poorly understood. Using miRanda for target prediction, Zhang et al. showed that miRNA-1258 targets heparanase (HPSE) which is the dominant endoglycosidase in mammals. HPSE favors tumor cell spread through dissolution of the extracellular matrix. In addition, this study introduced an inverse association between the miR-1258 levels and heparanase expression and enzymatic activity [53]. These findings were confirmed when the authors compared miRNA-1258 levels in clinical samples of invasive ductal carcinoma and BMBC compared with the corresponding normal or primary tissues. Moreover, the expression of miR-1258 in BM from breast cancer cells has been shown to suppress heparanase *in vitro* cell invasion and experimental BM.

5. The Blood-Brain-Barrier Is an Additional Challenge for BM Therapeutic Development

The BBB comprises a specialized endothelium surrounded by a thick basement membrane and astrocytic endfeet [48]. Compared with endothelial cells from other vascular beds, brain microvascular endothelial cells characteristically have very low permeability to solutes and hydrophilic molecules, high electrical resistance, complex tight junctions, and an array of metabolic and transport systems that supply the brain with nutrients and eliminate brain metabolic by-products. The low permeability is important in protecting the brain from circulating toxins and restricting the migration of leukocytes and monocytes, consequently it is also hindrance to drug access. The BBB endothelia specifically express efflux transporters that further diminish the brain availability of certain chemotherapeutic agents, including p-glycoprotein, breast cancer resistance p-glycoprotein, and multidrug resistance-associated protein [54]. Moreover, binding of specific plasma proteins to chemotherapeutic drugs has been shown to decrease the concentrations of such drugs and subsequently, delivery of these drugs to the brain [41]. Therefore, poor drug delivery across the BBB is thought to be a major factor underlying the limited efficiency of systemic chemotherapies against BM from breast cancer, particularly monoclonal antibodies like Trastuzumab [48]. Large hydrophilic molecules, such as chemotherapeutic and molecular-targeted drugs, are excluded from the central nervous system unless they can be actively transported by receptor-mediated transcytosis. This highlights the need for new brain-permeable drugs.

In order to metastasize to the brain, breast cancer cells must attach to and invade through the BBB. A widely supported hypothesis is that breast tumour cell adhesion induces retraction of the endothelium, which exposes the vascular basement membrane to the breast cancer cells. There is ample evidence that breast cancer cells recognize and bind to components in the vascular membrane, thereby initiating extravasation and the beginning of colonization at secondary organ sites [43]. The impairment of the BBB was observed recently in breast cancer patients who developed metastasis to the brain. CXCR4/SDF-1α has been suggested to play a major role in penetration of breast cancer cells through brain microvascular endothelial cells. In this regard, it has been reported that SDF-1-α-mediated blood vessel instability via

enhanced vascular permeability facilitates BM from breast cancer in a PI3K/Akt dependent manner [35].

The characteristics of the BBB necessitate the use of specialized, biologically relevant (and preferably throughput) preclinical models to test the efficacy of therapeutics against BM. Neural-cancer cell co-cultures can be used to simulate a brain-like microenvironment *in vitro* [55], but these simple models do not test whether compounds could cross the BBB *in vivo*. Bos and colleagues have developed a more biologically relevant *in vitro* transmigration assay, in which cancer cells adhere to and migrate across a human umbilical vein endothelial cell barrier towards human astrocytes segregated in a transwell chamber [34]. The gold standard preclinical model would involve xenograft transplantation of human BM into immunocompromised mice, but development is limited by the availability of fresh human tissue, and to our knowledge, none have been developed using metastatic cells selected in the human brain microenvironment. Alternative models have been generated by *in vivo* selection of neurotropic clones in nude mice, following intracardiac injection of metastatic cells from breast (231-BR, MCF7-HER2-BR, CN34-BM, and the mouse mammary tumour-derived 4T1 syngeneic model) [56, 57]. Table 1 summarizes a series of genes derived from integrated studies that have been implicated in BM development.

6. Treatment Strategies against Metastatic Breast Cancers Including Brain Metastases

We are approaching an era of personalized medicine, where therapies will be routinely tailored to individual tumours based on molecular diagnostics. Several targeted therapies for metastatic breast cancer are currently used or in clinical trials (Table 2; examples discussed below).

A recent phase II clinical trial using Lapatinib (dual inhibitor of HER1/2 with good brain penetration) in HER2+ breast cancer patients with BM despite prior radiation and Trastuzumab therapy showed Lapatinib has modest CNS antitumour activity [68]. This was corroborated in a mouse model where Lapatinib inhibited the colonisation of 231BR cells overexpressing EGFR and HER2 [48]. There is also evidence of synergism with Capecitabine, a pyrimidine analogue prodrug approved for metastatic breast cancer management [41, 54]. Sunitinib, a multikinase inhibitor (targets include VEGF-Rs, c-kit and PDGF-Rs), is also under investigation for its effects on BM in metastatic breast and renal cell carcinomas [64, 65, 69].

7. Possible "Druggable" Targets for the Future in BM from Breast Cancer

A recent study has demonstrated the role of *STAT3* in BM from breast cancer [38]. Moreover, it has been shown that *STAT3* inhibition by restoration of its inhibitor, suppressor of cytokine signalling (SOCS-1), results in induction of Caveolin-1, a tumour suppressor gene in breast cancer [38]. In this regard, STAT3 might be an attractive therapeutic target in BM from breast cancer.

TABLE 2: Current targeted therapies and their use in metastatic breast cancer (MBC).

Drug	Class	Targets	Clinical indications	Reference
Trastuzumab	mAb	HER2	Single agent for HER2+ MBC; used in combination with paclitaxel as first-line therapy for HER2+ MBC	[58]
Lapatinib	TKI	HER1/2	Active in Trastuzumab-resistant, HER2+ breast cancer; crosses the BBB and suppresses CNS metastasis used in combination with Capecitabine for HER2+ MBC	[41]
Pertuzumab	mAb	HER2	Impairs HER2 homo-/hetero-dimerisation; active in Trastuzumab-resistant HER2+ breast cancers; not currently approved for MBC	[59]
Neratinib	TKI	HER1/2	Inhibits HER2 autophosphorylation and suppresses downstream signalling; active in HER2+ patients with and without Trastuzumab pretreatment; not currently approved for MBC	[60]
Bevacizumab	mAb	VEGF	Antiangiogenic therapy for MBC in combination with Docetaxel or Paclitaxel for first-line treatment	[61]
Sorafenib	TKI	VEGFR PDGFR Raf	Multitarget receptor tyrosine kinase inhibitor; FDA-approved for advanced renal cancer and hepatocellular carcinoma	[62]
Axitinib	TKI	VEGFR PDGFR	Currently in phase III clinical trial for metastatic renal cell carcinoma	[63]
Sunitinib	TKI	VEGFR PDGFR KIT	Antiangiogenic therapy; FDA-approved for renal cell carcinoma and Gleevec-resistant gastrointestinal stromal tumours; effective as a single agent for metastatic breast cancer (phase II study)	[64–66]
Pazopanib	TKI	VEGFR PDGFR KIT	Anti-angiogenic therapy; FDA-approved for renal cell carcinoma; combination with Lapatinib has superior activity as the first-line treatment for MBC (phase II study)	[67]

Abbreviations: BBB: blood-brain barrier; CNS: central nervous system; HER1: Epidermal Growth Factor Receptor (EGFR); mAb: monoclonal antibody; KIT: Mast/stem cell growth factor receptor gene; PDGFR: platelet-derived growth factor receptor; TKI: tyrosine kinase inhibitor; VEGFR: vascular endothelial growth factor receptor.

Secondly, a growing body of evidence suggests that heparanase, a downstream target of EGFR/HER2, might be involved in BM from breast cancer [53]. EGF induces nucleolar translocation of HPSE and induction of DNA topoisomerase I which is essential for BM from breast cancer and cell proliferation [53]. In this setting, heparanase has been suggested as a potential target that could be exploited therapeutically [53].

Palmieri et al. [49] reported that hexokinase 2 (HK2) is significantly increased in brain metastases compared to unmatched primary breast tumors. Hexokinases phosphorylate glucose to produce glucose-6-phosphate in the first step of glucose metabolism. It is thought that HK2 increases glycolysis in tumor cells and may therefore be an attractive therapeutic target [49].

We recently identified somatic activating mutations in genes associated with the AKT and MAPK signaling pathways, including *PIK3CA*, *KRAS*, *HRAS*, and *NRAS*, in BM from breast and other cancers [12]. This highlights the possibility of cancer cells resisting targeted treatment to molecules such as HER2 or EGFR by acquiring oncogenic mutations in downstream pathways. Therapeutic modalities targeting these downstream pathways are currently being investigated [59]. In addition, Grigoriadis et al. demonstrated the presence of CT-X antigens in a cohort of BM, including BM from

breast cancer. These proteins are predominantly expressed in human germ cells and not somatic tissues, but are frequently activated in cancer. Nearly two-thirds of our BM cohort showed expression of MAGE-A and NY-ESO, two of the CT-X antigens for which inhibitors are currently in clinical trials for lung cancer and melanoma [70]. Targeting these antigens could therefore also be an effective therapeutic strategy for BM.

8. Future Directions: Brain Metastases in the Whole Genome Sequencing Era

Direct, high-resolution genomic and transcriptomic analyses of BM may uncover new druggable features that can be targeted to specifically inhibit BM cells. The development and continual refinement of WGS technologies has dramatically increased the resolution with which we can analyse cancer genomes. We can now screen for new mutations, simultaneously and accurately assess their frequencies and correlate this with expression data across the entire genome. Rare mutations, that are present in only a small percentage of cells within the heterogeneous primary tumour mass, may be important in progression and virulence acquisition at a metastatic site. Although there are no large-scale BM genomics studies published to date, Ding and colleagues used WGS

to interrogate the genomic profiles of a human BM, its corresponding primary breast tumour, and a mouse xenograft derivative [44]. They showed that; (1) most of the genomic changes present in the primary tumour are propagated during the clinical course of the disease; (2) the frequency of some mutations was increased in the BM, suggesting enrichment with a subpopulation of cells selected from a more heterogeneous primary tumour; (3) two different populations of cells from the primary tumour, with distinct subsets of mutations, were selected during the metastatic process. The third finding is striking and is consistent with other data suggesting that metastases may be established from clusters of cells, rather than one founder cell [71] or may derive from regions of the tumour where there is clonal heterogeneity [16].

9. Conclusions

Thus far, we have only fragmented knowledge of the mechanisms underlying breast cancer cell colonisation of the brain microenvironment. Animal models and gene expression profiling have and will continue to provide insight, however it is anticipated that next-generation sequencing technology and integration of this data with expression profiling will enable us to generate a comprehensive map of the brain metastasis genomic landscape. To be able to translate this impending knowledge into clinical outcomes, we must continue to develop and refine high-throughput, biologically relevant preclinical models that accurately mimic the brain microenvironment and the BBB (summarized in Figure 1).

Acknowledgments

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Research Article

Integrated Genomic Analysis of Sézary Syndrome

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Sézary syndrome (SS) is a rare variant of primary cutaneous T-cell lymphoma. Little is known about the underlying pathogenesis of S. To address this issue, we used Affymetrix 10K SNP microarray to analyse 13 DNA samples isolated from 8 SS patients and qPCR with ABI TaqMan SNP genotyping assays for the validation of the SNP microarray results. In addition, we tested the impact of SNP loss of heterozygosity (LOH) identified in SS cases on the gene expression profiles of SS cases detected with Affymetrix GeneChip U133A. The results showed: (1) frequent SNP copy number change and LOH involving 1, 2p, 3, 4q, 5q, 6, 7p, 8, 9, 10, 11, 12q, 13, 14, 16q, 17, and 20, (2) reduced SNP copy number at FAT gene (4q35) in 75% of SS cases, and (3) the separation of all SS cases from normal control samples by SNP LOH gene clusters at chromosome regions of 9q31q34, 10p11q26, and 13q11q12. These findings provide some intriguing information for our current understanding of the molecular pathogenesis of this tumour and suggest the possibility of presence of functional SNP LOH in SS tumour cells.

1. Introduction

Sézary syndrome (SS) is a rare subset of primary cutaneous T-cell lymphoma (CTCL) with an aggressive clinical course [1, 2]. SS typically presents with generalised skin lesions or erythroderma, intense pruritus, and peripheral lymphadenopathy. A blood test will reveal atypical T-lymphocytes with convoluted nuclei, Sézary cells, which are often associated with a cutaneous T-cell lymphoma [2–5]. Recent studies have revealed complex genetic aberrations affecting almost all chromosomes [6–14] with loss of heterozygosity (LOH), [9, 15–19] and deregulation of several genes, [20–26] and epigenetic changes [27, 28] in SS. Despite this progress, the key molecular targets underlying the pathogenesis of this type of skin lymphoma remain elusive.

Single nucleotide polymorphism (SNP) denotes a single base in the DNA sequence that differs from the usual base at that position. Millions of SNPs have been catalogued in the human genome, which may be responsible for disease such as cancer (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp).

SNP microarray is a powerful genome research technique designed for the identification of SNP and dose change within the whole genome [29–31]. This method has been widely used to investigate SNP copy number change and LOH in cancer genome in a variety of medical and biological subjects [32–42]. However, there has been no report describing the use of SNP microarray to assess genomewide SNP copy number change and LOH in SS in the literature (http://www.ncbi.nlm.nih.gov/sites/entrez).

To address this issue we initially performed SNP microarray on DNA samples isolated from biopsies of skin lesions and peripheral blood mononuclear cells (PBMCs) of patients with SS by using Affymetrix GeneChip Mapping 10K Xba assay. Then we assessed gene-specific SNP copy number changes in same DNA samples with ABI TaqMan SNP genotyping assays to compare and verify the findings from Affymetrix 10K SNP microarray analysis. Finally, we conducted a literature review on LOH findings of patients with SS. Our findings are presented here in this paper.
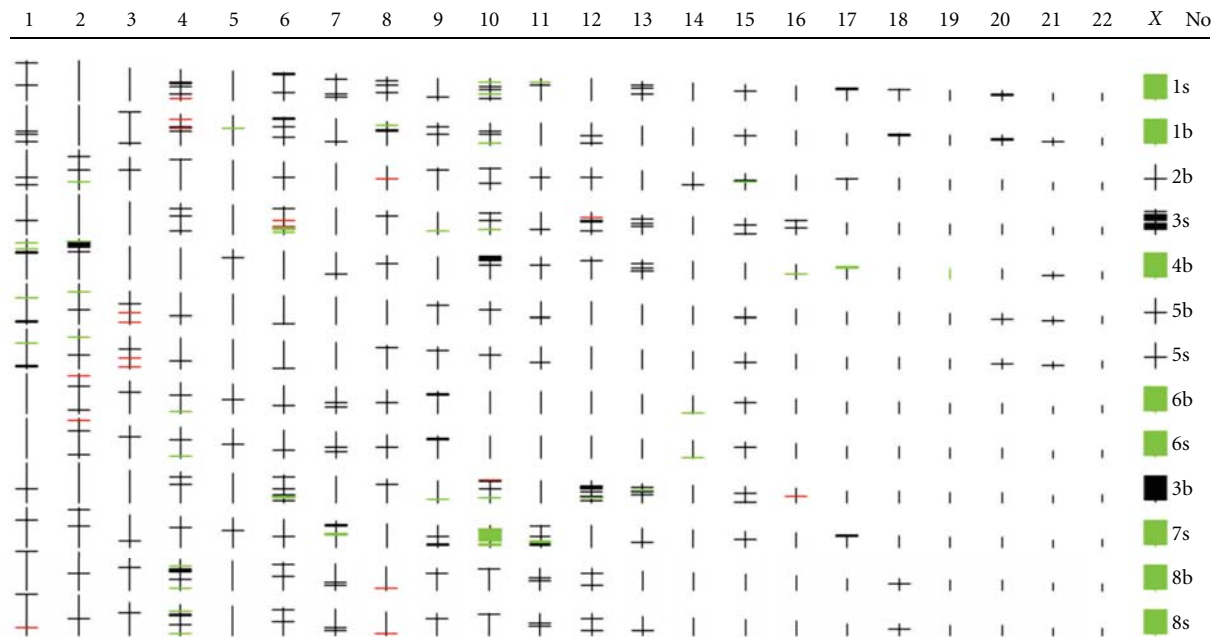
FIGURE 1: Illustration of a diagram of genomewide SNP homozygosity mapping in 8 SS cases (No. 1–8) detected with Affymetrix GeneChip Human Mapping 10K Array (http://www.affymetrix.com/products/arrays/specific/10k.affx) and analysed by using GOLF software (https://bioinformatics.cancerresearchuk.org/cazier01). Here each red-coloured line represents copy number gain of individual SNP, green-coloured line and block stand for SNP copy number loss, black-coloured line and block denote balanced/neutral SNP copy number or uniparental disomy. Similar SNP homozygosity profile is present in paired DNA samples from the biopsied skin lesion (S) and peripheral blood (B) isolated from individual patient with SS on the right end of the diagram. Frequent SNP homozygous changes (>3) are seen on almost all autosomes consistent with SNP copy number alterations shown in Figure 2. The remarkable changes on X chromosome were used as the internal quality control for the 10K SNP array experiment as green represents X chromosome copy number loss or monosomy indicating male sex, and black signifies balanced or disomy X chromosome denoting female.

2. Materials and Methods

2.1. Sample Selection and DNA Preparation.
Eleven patients with Sézary syndrome were selected for this study according to the World Health Organization and the European Organization for Research and Treatment of Cancer diagnosis criteria for CTCL [2]. Genomewide SNP analysis was performed on 8 of these SS cases using Affymetrix SNP microarray. Five of these 8 SS cases plus additional 3 cases were further assessed with SNP quantitative real-time PCR in this study. Briefly, a total of 16 DNA samples (13 samples for SNP microarray and 3 additional samples for SNP qPCR), which were taken prior to treatment, were extracted from both PBMCs and biopsied skin lesions from these SS cases (Figures 1 and 2). This was conducted by using QIAGEN Genomic-tip 100/G kit (QIAGEN Ltd, West Sussex, UK) according to the manufacturer's instruction (http://www1.qiagen.com/). DNA concentrations and purity were determined with the NanoDrop ND-1000 spectrophotometer (NanoDrop, Rockland, Del, USA). Local ethical approval for the sampling procedures was obtained previously [9–11, 18] and the use of the stored DNA samples for this study was approved by the Research Ethical Committee at Barts and Royal London School of Medicine and Dentistry, Queen Mary University of London.

2.2. Single Nucleotide Polymorphism Microarray Assay.
Affymetrix SNP microarray analysis of 13 DNA samples including 5 paired PBMCs and skin biopsies from 8 SS patients (Figure 1) was conducted according to the standard GeneChip Mapping 10 K (V2.0) Xba Assay protocol (Affymetrix Inc., Santa Clara, Calif, USA). Briefly, 350 ng of DNA was digested with XbaI and ligation to the XbaI adaptor prior to PCR amplification (35 cycles) using AmpliTaq Gold with Buffer II (Applied Biosystems, Foster City, Calif, USA). Hybridised arrays were processed with an Affymetrix Fluidics Station 450 and fluorescence signals were detected using the Affymetrix GeneChip Scanner 3000. Signal intensity data was assessed with the GeneChip DNA analysis software (http://www.affymetrix.com/products/arrays/specific/10k.affx) based on a model algorithm to generate SNP calls [43] and subsequent bioinformative analysis was carried out using an in-house software called GOLF (http://www.bioinformatics.cancerresearchuk.org/cazier01) developed by Professor Young [44] and a publicly accessible software named IdeogramBrowser Software (http://www.informatik.uni-ulm.de/ni/staff/HKestler/ideo/doc.html).

2.3. SNP Quantitative Real-Time PCR Assay.
To verify the results of Affymetrix SNP microarray analysis of SS cases in this study, SNP qPCR assay was performed to test 10 DNA samples from 8 SS patients including 7 samples from 5 SS cases used in above SNP microarray and 3 samples from additional 3 SS cases (Figure 3). This experiment was carried out by means of an ABI Prism 7900 sequence detection
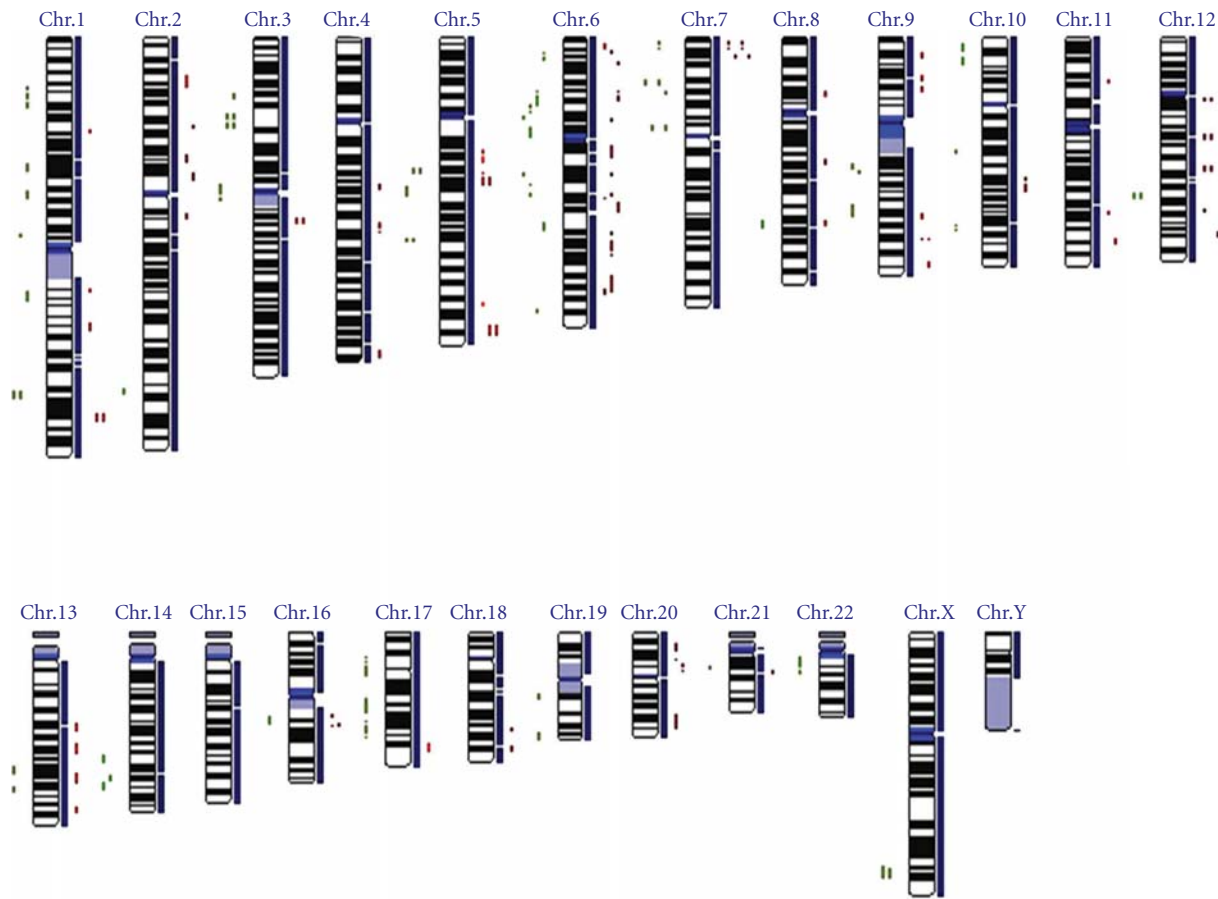
FIGURE 2: Illustration of an ideogram of genomewide SNP copy number changes in 8 SS cases identified by using Affymetrix GeneChip Human Mapping 10K Array as above and analysed with the IdeogramBrowser Software (http://www.informatik.uni-ulm.de/ni/staff/HKestler/ideo/doc.html). Here each red-coloured dot represents copy number loss of individual SNP and green-coloured dot stands for SNP copy number gain. Frequent SNP copy number changes at chromosomes 1, 2p, 3, 4q, 5q, 6, 7p, 8, 9, 10, 11, 12q, 13, 14, 16q, 17, and 20 are clearly visible.

Gene	SNP	TaqMan SNP genotyping assay ID	Location	SS1a	SS1b	SS3	SS5a	SS5b	SS6	SS8	SS9	SS10	SS11
RAB1A	rs10519011	C_30505234_20	2p14			red	green						green
STAT4	rs13001658	C_26419547_10	2q32.2q32.3			green	green						
VEGFC	rs1485765	C_8850347_20	4q34.1q34.3			red	green		red	red	red	yellow	green
FAT	rs1280100	C_11250338_10	4q35	red		red	yellow	yellow	red	red	red		yellow
TWIST1	rs2106892	C_26517452_10	7p21.2			green	green						
BNIP3L	rs1042992	C_8796859_20	8p21			yellow	green						
NFIB	rs2078463	C_16110577_10	9p24.1		red								
AKR1C3	rs7068685	C_11654325_20	10p15p14	green		red	yellow	green	green	red	yellow	yellow	green
TAGLN	rs2269397	C_26872972_10	11q23.2			green	green		green				
NFIB	rs2382446	C_27086526_10	chr12			green	red		red	red			
TRIM16	rs9909923	C_30222562_10	17p11.2			red	yellow	green	red	red	yellow		
PLS3	rs5987755	C_30587612_20	Xq23			red	green						green

FIGURE 3: A summary of SNP copy number changes of 12 genes in 10 SS samples by using qPCR with ABI TaqMan SNP genotyping assay to verify the results of Affymetrix 10K SNP microarray analysis of SS in this study. Here red-coloured rectangular bar denotes SNP copy number loss against internal controls of B2M and GAPDH, green-coloured bar stands for SNP copy number gain, yellow-coloured bar represents balanced/neutral or normal SNP copy number, and gray-coloured bar indicates noninformative. Frequent SNP copy number losses of VEGFC, FAT, NFIB, and TRIM16 are clearly visible.

system using the TaqMan PCR Master Mix with TaqMan SNP genotyping assays (http://www3.appliedbiosystems.com/applicationstechnolo-gies/real-timepcr/index.htm). These assays amplify 12 genes within the chromosomal regions showing frequent SNP copy number changes identified in this study (Figure 2). Additional 2 SNP genotyping assays amplifying beta-2-microglobulin (B2M, rs935885, C_12080829_10) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, rs3741918, C_27510362_10) were used as the internal quality controls. qPCR reactions contained 900 nmol primers, 200 nmol probes, 400 mM each dATP, dCTP and dGTP, 800 mM dUTP, 1 U Amplitaq Gold DNA polymerase, 0.2 U AmpErase uracil N-glycosylase (UNG), and 13 TaqMan buffer in a total volume of 25 μL in 96-well microtiter plate (Applied Biosystems). After a decontamination step at 50°C, a two-step protocol was followed for 50 cycles: 95°C for 15 s and 61°C for 1 min. SNP qPCR data analysis was conducted as previously described [45] with ABI SNPbrowser Software (http://marketing.applied-biosystems.com/mk/get/snpb_landing).

2.4. SNP Loss of Heterozygosity Expression Profiling. We have previously put forward a concept of functional copy number changes in cancer cells [46]. To further test our hypothesis in this study, we assume a SNP LOH gene cluster in SS is bioinformatically significant or functional if it is capable of separating all SS cases from normal control (NC) samples. This test was carried out through a data mining experiment by using a bioinformative method developed by us [46]. In this experiment, the effect of SNP LOH detected in this study on gene expression profiling in SS cases conducted in previous studies [23, 24, 47] was tested as before [46]. The procedures were briefly described as follows. We initially created gene lists from 9 chromosomal regions showing frequent LOH (>3 per region) at SNP level seen in SS (Table 1) by using IdeogramBrowser Software and UCSC Human Gene Sorter (http://www.genome.ucsc.edu/). Then we imported these 9 gene-lists into GeneSpring software version 7 (http://www.sigenetics.com/), which contained a genome/raw data of gene expression profile from 6 SS patients who were treated with photopheresis therapy and 2 healthy individuals generated by using Affymetrix GeneChip U133A (http://www.affymetrix.com/products/arrays/specific/hgu133av2.affx). Of 6 SS cases analysed with Affymetrix gene expression microarray the 3 cases were also tested by above Affymetrix SNP microarray. Finally, we analysed the selected 9 gene lists by using the clustering tool of GeneSpring to generate gene tree and condition tree, a supervised clustering, which enables direct visualisation of sample congregation and separation [46].

2.5. Comparison with Published Data on LOH at Microsatellite Loci in SS. The genomic region where SNPs span on Affymetrix 10K GeneChip used in this study is equivalent to that covered by 400 microsatellite (MS) loci (http://www.affymetrix.com/products/arrays/specific/10k.affx). There have been several publications describing LOH at MS loci on limited chromosomes in CTCL including SS [9, 15–19, 48].

Table 1: A summary of gene expression profile of LOH gene clusters.

Chromosomal regions with LOH detected in this study	Number of genes	Separation of SS from NC*
1p36p33	80	N
2q21q24	9	N
8q22q24	15	N
9q31q34	40	P
10p11q26	111	P
11q21q25	205	N
13q11q12	15	P
16q21q23	74	N
17p13q11	198	N

*N: no separation between SS and NC, P: presence of separation between SS and NC.

To compare previous findings with this study, we reanalysed previous published data on MS LOH at chromosomes 1 and 10q through fine mapping of each MS locus, which was linked to individual gene and SNP by using NCBI ENTREZ GENE and ENTREZ SNP database (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. SNP Microarray. In this study, paired DNA samples from PBMCs and skin lesions from 5 of 8 SS cases were analysed with Affymetrix 10 K SNP microarray, which showed similar SNP homozygous changes between the paired samples indicating they are coming from the same individual. There was a difference in SNP fingerprint among 8 different SS cases signifying the absence of sample contamination. As Figure 1 shows, frequent homozygous changes (>3 per chromosome) including gain, loss, and uniparental disomy (UPD) were seen to distribute throughout almost an entire genome except of chromosomes 14, 19 and 22.

As Figure 2 shows there were frequent SNP copy number changes (>3 per chromosome) at chromosomes 1, 2p, 3, 4q, 5q, 6, 7p, 8, 9, 10, 11, 12q, 13, 14, 16q, 17, and 20, which are not only consistent with above homozygous changes but also in line with previous CGH findings in SS [10].

In this study, a weak and/or smear band of TCR gene was detected in skin DNA samples, while a strong monoclonal TCR gene band was seen in PBMC DNA samples (data not shown), suggesting that only a few tumour cells were present in skin samples and sample contamination was negligible. Overall, a frequent LOH was noted in 5 paired SS cases at 9 chromosomal regions including 1p36p33, 2q21q24, 8q22q24, 9q31q34, 10p11q26, 11q21q25, 13q11q12, 16q21q23, and 17p13q11 (Table 1), which are to some extent similar to previous reports on LOH at certain chromosome regions [9, 15–19].

3.2. SNP qPCR Assay. To further confirm SNP microarray findings, SNP qPCR was utilised to test DNA samples from 5 of 8 SS cases (case number 1, 3, 5, 6 and 8) analysed with

Affymetrix 10K SNP microarray using ABI TaqMan SNP genotyping assay (63%) and 3 additional cases. As Figure 3 shows, frequent SNP copy number loss (>2 cases) at the FAT gene (rs1280100) at 4q35 was observed in 6 of 8 SS cases (number 1, 3, 6, 8, 9, and 10) (75%), followed by VEGFC (rs1485765) at 4q34.1q34.3 (cases 3, 6, 8, and 9) (50%), NFIB (rs2382446) at chromosome 12 (cases 5, 6 and 8) (38%), and TRIM16 (rs9909923) at 17p11.2 (cases 3, 6, and 8) (38%). While frequent SNP copy number gain (>2 cases) was also noted at the AKR1C3 gene (rs7068685, 50%), VEGFC (rs1485765, 38%), and TAGLN (rs2269397, 38%).

SNP copy number changes were most frequent present in SS case 3, which showed losses of genes of RAB1A (rs10519011, 2p14), VEGFC, **FAT**, AKR1C3, TRIM16, and PLS3 (rs5987755, Xq23) as well as gains of genes of TAGLN (rs2269397, 11q23.2) and NFIB. This was followed by case 5, which had gains of RAB1A, STAT4 (rs13001658, 2q32.2q32.3), VEGFC, TWIST1 (rs2106892, 7p21.2), TAGLN, and PLS3, and loss of NFIB. Case 6 revealed losses of VEGFC, **FAT**, NFIB and TRIM16, and gain of AKR1C3; case 8 demonstrated losses of VEGFC, **FAT**, AKR1C3, NFIB, and TRIM16 (Figure 3). Overall these SNP copy number changes, detected by using qPCR with ABI TaqMan SNP genotyping assay, were consistent with those identified by using Affymetrix 10K SNP microarray as shown in Figure 2.

3.3. SNP LOH Expression Profiling. The impact of SNP LOH gene cluster on gene expression pattern was further tested in this study. As shown in Figure 4, the clusters of 2 NCs appeared at the right end of the heatmap and the clusters of 6 SS cases were present in the middle and at the left end of the heatmap. This clearly showed that two groups were separated and different. Over the 9 SNP LOH gene lists analysed in this study, 3 (33%) showed gene expression patterns of the separation of 6 SS cases from 2 NCs (Table 1). This included SNP LOH gene clusters at 9q31q34 (40 genes), 10p11q26 (111 genes), and 13q11q12 (15 genes) (Figure 4). Each individual gene in these 3 gene clusters is listed in Table 2. However, the remaining 6 SNP LOH gene lists revealed a mixed gene expression pattern without the separation of SS from NCs (Table 1).

3.4. Refining Previous SS LOH Data and Comparing with This Study. Previous studies have shown LOH on several chromosomes including chromosomes 1 and 10q [9, 15–19, 48]. The 10K SNP microarray gene chip used in this study is equivalent to the genetic distance covered by 400 MS. This allows us to compare SNP LOH identified in this study with MS LOH described in those previous studies. Frequent LOH at MS loci D1S247 (rs11372930) at 1p36 and D10S562 (rs4128597) at 10q25.3 (Figure 5) reported in previous studies [9, 18, 19] were seen in this study.

4. Discussion

This integrated genomic study has for the first time revealed genomewide SNP copy number change and LOH as well as SNP LOH gene expression profiling in SS cases. Although

the number of SS cases tested in this study was small, the patterns of SNP copy number change and LOH identified in SS cases are generally in line with previous metaphase-/array-CGH [6–14] and MS LOH studies [9, 15, 17–19, 48], suggesting the high efficiency and reliability of the methods used. In addition some novel findings emerged. This includes the presence of SNP copy number loss at the FAT gene (4q35) in most SS cases and the separation of all SS cases from NC samples by SNP LOH gene clusters at chromosome regions of 9q31q34, 10p11q26, and 13q11q12. Although all of these results will need to be validated in larger cohorts of samples from SS patients in the future, they do provide some intriguing information for our current understanding of the molecular pathogenesis of this type of lymphoma and suggest the possibility of presence of functional SNP LOH in SS tumour cells at least from the point of view of bioinformatics.

Sézary syndrome is a rare form of CTCL with an aggressive clinical course and it also likely represents the leukaemic phase of mycosis fungoides [1, 2]. Like any other advanced stage of malignancies, SS accumulates a variety of genetic and epigenetic events including alterations of tumour suppressor genes, oncogenes, and some key house-keeping genes [18, 27, 28, 49]. Previous studies on SS have shown complex genetic aberrations affecting almost all chromosomes with copy number losses being the dominant abnormalities, which mainly involve chromosomes 1p, 6q, 9, 10, 13q, and 17p [6–14] and the presence of LOH at MS loci on chromosomes 1p, 9p, 10q, and 17p in tumour cells [9, 15–19, 48]. In addition, downregulation of several genes including MXI1 (10q24q25) and upregulation of genes such as TWIST1 (7p21.2) and PLS3 (Xq23) have also been described in this malignancy [20–26]. Similar results were obtained in this study, which showed SNP copy number change on chromosomes 1, 2p, 3, 4q, 5q, 6, 7p, 8, 9, 10, 11, 12q, 13, 14, 16q, 17, and 20, and LOH at 1p, 2q, 8q, 9q, 10, 11q, 13q, 16q, and 17 as well as SNP copy number gains of TWIST1 and PLS3 in 2 SS cases, further indicating the high efficiency and reliability of the integrated genomic experiments conducted in this study.

Although there are different definitions for loss of heterozygosity in literature, LOH is generally defined as a locus or loci at which a deletion or other process has converted the locus from heterozygosity to homozygosity or hemizygosity. In terms of cancer, LOH signifies that in tumour cells carrying a mutated allele of a tumour suppressor gene, the gene becomes fully inactivated when the cell loses a large part of the chromosome carrying the wild-type allele (http://www.ncbi.nlm.nih.gov/cancerchromosomes). LOH is traditionally used as a molecular tool to identify a tumour-suppressor gene but it also represents one of the most common molecular features of cancer cells [47, 50–52]. Previous studies have shown LOH at MS loci on chromosomes 1p, 9p, 10q, 12q, 13q, 17p, and 19 in CTCL including SS [9, 15–19, 48]. This was supported by previous conventional cytogenetics and metaphase and array-based CGH studies, which revealed DNA copy number losses at these chromosomal regions [6–14, 48]. In this study LOH at SNP level on 9 chromosomal regions (1p, 2q, 8q, 9q, 10, 11q, 13q, 16q, and 17)

15 genes from 13q loh gene list SSvcontrol (8 samples)

36830_at(MIPEP)
218479_s_at(XPO4)
208742_s_at(SAP18)
208740_at(SAP18)
204386_s_at(MRP63)
204305_at(MIPEP)
213262_at(SACS)
204387_x_at(MRP63)
221995_s_at(MRP63)
208741_at(SAP18)
221771_s_at(HSMPP8)
220753_s_at(CRYL1)
206404_at(FGF9)
208590_x_at(GJA3)
207302_at(SGCG)
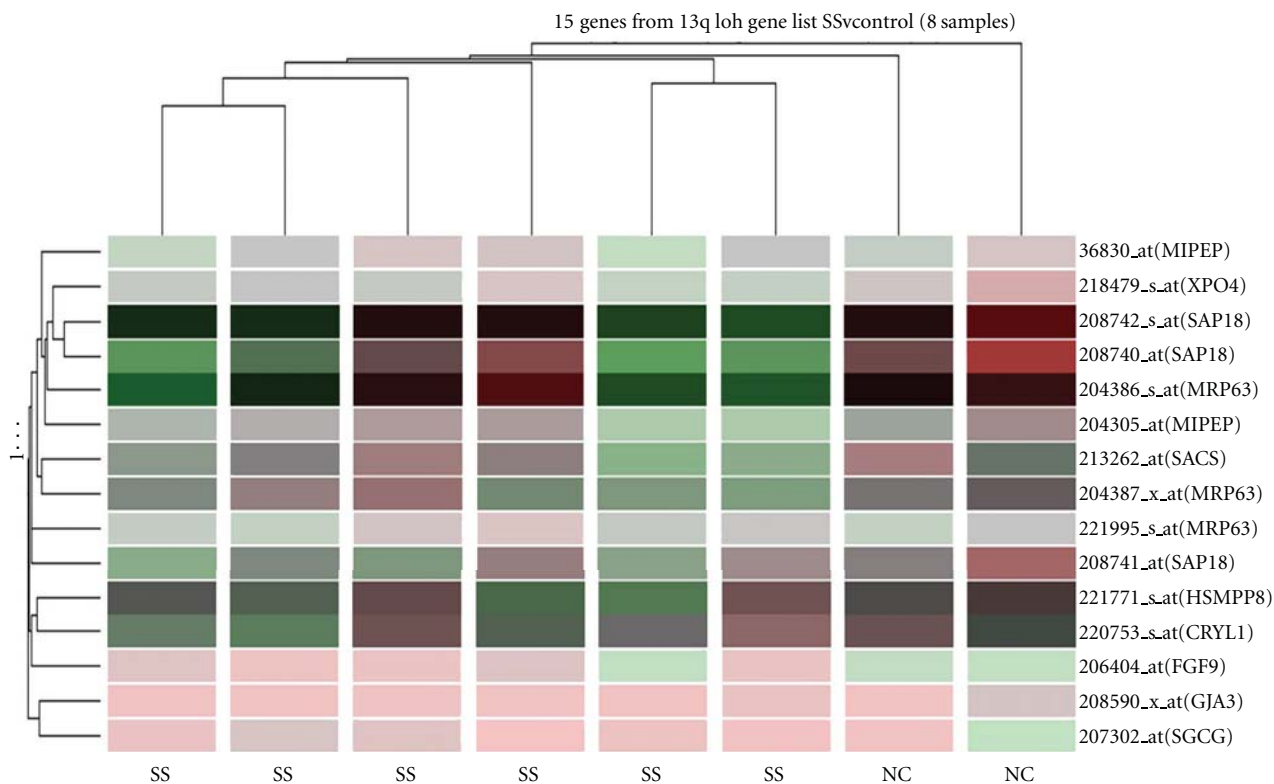
SS   SS   SS   SS   SS   SS   NC   NC

Figure 4: Illustration of a heatmap of gene expression profile of SNP LOH at 13q11q12 gene cluster (15 genes). Here each column represents one test sample and each coloured rectangular bar signifies each individual gene. The colouration of each bar indicates the expression level of individual gene, which has been reported previously [23, 24, 47] and is not the focus of this study. The separation of 6 Sézary syndrome cases (SS, left and middle) from 2 normal controls (NC, right) is clearly visible and two test groups are different.

MS Loci	Gene	SNP	Location
D10S583	CDC2	rs57066318	10q21.1
D10S581	EGR2	rs61865883	10q21.1
D10S541	BMPR1A	rs6586038	10q22.3
D10S192	HPS1	rs59684510	10q23.1q23.3
D10S215	PAPSS2	rs4934358	10q23q24
D10S571	SORBS1	rs4146488	10q23.3q24.1
D10S185	FER1L3	rs871422	10q24
D10S198	PAX2	rs11819541	10q24
D10S603	PKD2L1	rs3853518	10q24
D10S190	CYP17A1	rs45504696	10q24.3
D10S222	NEURL	rs11191726	10q25.1
D10S562	**TCF7L2**	**rs4128597**	**10q25.3**
D10S187	KIAA1598	rs7099910	10q25.3
D10S530	DMBT1	rs1960177	10q25.3q26.1
D10S221	PDZD8	rs2532837	10q25.3q26.11
D10S587	FGFR2	rs2981582	10q26
D10S544	HSPA12A	rs2907230	10q26.12
D10S209	PPAPDC1A	rs4752440	10q26.12

Sample columns: SS20, MF15, SS24, SS22, MF12, MF8, MF7, MF6, MF13, SS26, SS16, SS19, SS23, SS25, MF10, MF4, MF14, SS18, SS21, SS17, MF1, MF5, MF9, MF11, MF3, MF2

Figure 5: A summary of reanalysis of previously published LOH at 18 microsatellite loci on 10q21q26 in SS and MF [19] with this study, in which each microsatellite locus is finely mapped and linked to individual gene and SNP via NCBI ENTREZ GENE and ENTREZ SNP database (http://www.ncbi.nlm.nih.gov/). Here black bar represents LOH, gray bar stands for noninformative, and white bar indicates microsatellite marker that is not available. One SNP within microsatellite locus D10S562 (bold) is also present in the SNP LOH cluster at 10p11q26 in this study (Table 1).

TABLE 2: A detailed gene list of the 3 LOH gene clusters separating SS from NC

LOH gene clusters	Gene name	Common	Description
9q31q34	221294_at	GPR21	G-protein-coupled receptor 21
	221085_at	TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15
	220935_s_at	CDK5RAP2	CDK5 regulatory subunit associated protein 2
	220300_at	RGS3	Regulator of G-protein signalling 3
	220202_s_at	MNAB	Membrane-associated nucleic acid binding protein
	220201_at	MNAB	Membrane-associated nucleic acid binding protein
	219884_at	LHX6	LIM homeobox protein 6
	218941_at	FBXW2	F-box and WD-40 domain protein 2
	218489_s_at	ALAD	Aminolevulinate, delta-, dehydratase
	218487_at	ALAD	Aminolevulinate, delta-, dehydratase
	218160_at	NDUFA8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19 kDa
	215813_s_at	PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
	214465_at	ORM2	Orosomucoid 2
	214425_at	AMBP	Alpha-1-microglobulin/bikunin precursor
	214040_s_at	GSN	Gelsolin (amyloidosis, Finnish type)
	211503_s_at	RAB14	RAB14, member RAS oncogene family
	210825_s_at	STOM	Stomatin
	210824_at	STOM	Stomatin
	209162_s_at	PRPF4	PRP4 pre-mRNA processing factor 4 homolog (yeast)
	209161_at	PRPF4	PRP4 pre-mRNA processing factor 4 homolog (yeast)
	208828_at	POLE3	Polymerase (DNA directed), epsilon 3 (p17 subunit)
	208737_at	ATP6V1G1	ATPase, H+ transporting, lysosomal 13 kDa, V1 subunit G isoform 1
	205599_at	TRAF1	TNF receptor-associated factor 1
	205500_at	C5	Complement component 5
	205477_s_at	AMBP	Alpha-1-microglobulin/bikunin precursor
	205128_x_at	PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
	205127_at	PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
	205041_s_at	ORM1	Orosomucoid 1
	205040_at	ORM1	Orosomucoid 1
	204449_at	PDCL	Phosducin-like
	204448_s_at	PDCL	Phosducin-like
	203971_at	SLC31A1	Solute carrier family 31 (copper transporters), member 1
	203823_at	RGS3	Regulator of G-protein signalling 3
	203447_at	PSMD5	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 5
	201062_at	STOM	Stomatin
	201061_s_at	STOM	Stomatin
	201060_x_at	STOM	Stomatin
	200928_s_at	RAB14	RAB14, member RAS oncogene family
	200927_s_at	RAB14	RAB14, member RAS oncogene family
	200696_s_at	GSN	Gelsolin (amyloidosis, Finnish type)
10p11.21q26.3	32094_at	CHST3	Carbohydrate (chondroitin 6) sulfotransferase 3
	222072_at	ADD3	Homo sapiens clone DT1P1A11 mRNA, CAG repeat region
	221136_at	GDF2	Growth differentiation factor 2
	219963_at	DUSP13	Dual specificity phosphatase 13
	219957_at	RUFY2	RUN and FYVE domain containing 2
	219543_at	MAWBP	MAWD binding protein
	219509_at	MYOZ1	Myozenin 1

TABLE 2: Continued.

LOH gene clusters	Gene name	Common	Description
	218878_s_at	SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*)
	218871_x_at	GALNACT-2	Chondroitin sulfate GalNAcT-2
	218445_at	H2AFY2	H2A histone family, member Y2
	218249_at	ZDHHC6	Zinc finger, DHHC domain containing 6
	218046_s_at	MRPS16	Mitochondrial ribosomal protein S16
	218006_s_at	ZNF22	Zinc finger protein 22 (KOX 15)
	218005_at	ZNF22	Zinc finger protein 22 (KOX 15)
	216903_s_at	CBARA1	Calcium binding atopy-related autoantigen 1
	216345_at	KIAA0913	KIAA0913 protein
	216037_x_at	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
	216035_x_at	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
	214878_at	ZNF37A	Zinc finger protein 37a (KOX 21)
	214617_at	PRF1	Perforin 1 (pore forming protein)
	214508_x_at	CREM	cAMP responsive element modulator
	214366_s_at	ALOX5	Arachidonate 5-lipoxygenase
	214348_at	TACR2	Tachykinin receptor 2
	214338_at	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12
	214322_at	CAMK2G	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
	214136_at	NUDT13	Nudix (nucleoside diphosphate linked moiety X)-type motif 13
	213952_s_at	ALOX5	Arachidonate 5-lipoxygenase
	213647_at	DNA2L	DNA2 DNA replication helicase 2-like (yeast)
	212894_at	SUPV3L1	Suppressor of var1, 3-like 1 (*S. cerevisiae*)
	212762_s_at	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
	212761_at	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
	212759_s_at	TCF7L2	Transcription factor 7-like 2 (T-cell specific, HMG-box)
	212757_s_at	CAMK2G	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
	212669_at	CAMK2G	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
	212594_at	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
	212593_s_at	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
	212359_s_at	KIAA0913	KIAA0913 protein
	212322_at	SGPL1	Sphingosine-1-phosphate lyase 1
	212321_at	SGPL1	Sphingosine-1-phosphate lyase 1
	211809_x_at	COL13A1	Collagen, type XIII, alpha 1
	211668_s_at	PLAU	plasminogen activator, urokinase
	211343_s_at	COL13A1	Collagen, type XIII, alpha 1
	210956_at	PPYR1	Pancreatic polypeptide receptor 1
	210671_x_at	MAPK8	Mitogen-activated protein kinase 8
	210588_x_at	HNRPH3	Heterogeneous nuclear ribonucleoprotein H3 (2H9)
	210477_x_at	MAPK8	Mitogen-activated protein kinase 8
	210318_at	RBP3	Retinol binding protein 3, interstitial
	210171_s_at	CREM	cAMP responsive element modulator
	210110_x_at	HNRPH3	Heterogeneous nuclear ribonucleoprotein H3 (2H9)
	209910_at	SLC25A16	Solute carrier family 25 (mitochondrial carrier; Graves disease autoantigen), member 16
	209869_at	ADRA2A	Adrenergic, alpha-2A-, receptor
	209860_s_at	ANXA7	Annexin A7
	209834_at	CHST3	Carbohydrate (chondroitin 6) sulfotransferase 3
	209817_at	PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)

TABLE 2: Continued.

LOH gene clusters	Gene name	Common	Description
	209538_at	ZNF32	Zinc finger protein 32 (KOX 30)
	209457_at	DUSP5	Dual specificity phosphatase 5
	209045_at	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble
	208990_s_at	HNRPH3	Heterogeneous nuclear ribonucleoprotein H3 (2H9)
	208770_s_at	EIF4EBP2	Eukaryotic translation initiation factor 4E binding protein 2
	208769_at	EIF4EBP2	Eukaryotic translation initiation factor 4E binding protein 2
	208535_x_at	COL13A1	Collagen, type XIII, alpha 1
	208453_s_at	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble
	208381_s_at	SGPL1	Sphingosine-1-phosphate lyase 1
	208252_s_at	CHST3	Carbohydrate (chondroitin 6) sulfotransferase 3
	208152_s_at	DDX21	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 21
	208095_s_at	CAMK2G	Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma
	207965_at	NEUROG3	Neurogenin 3
	207630_s_at	CREM	cAMP responsive element modulator
	207543_s_at	P4HA1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I
	207347_at	ERCC6	Excision repair cross-complementing rodent repair deficiency, complementation group 6
	207127_s_at	HNRPH3	Heterogeneous nuclear ribonucleoprotein H3 (2H9)
	206747_at	KIAA0514	KIAA0514 gene product
	206261_at	ZNF239	Zinc finger protein 239
	206159_at	GDF10	Growth differentiation factor 10
	206010_at	HABP2	Hyaluronan binding protein 2
	205882_x_at	ADD3	Adducin 3 (gamma)
	205879_x_at	RET	Ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschsprung disease)
	205479_s_at	PLAU	Plasminogen activator, urokinase
	204446_s_at	ALOX5	Arachidonate 5-lipoxygenase
	204445_s_at	ALOX5	Arachidonate 5-lipoxygenase
	204120_s_at	ADK	Adenosine kinase
	204119_s_at	ADK	Adenosine kinase
	203916_at	NDST2	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2
	203666_at	CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
	203187_at	DOCK1	Dedicator of cyto-kinesis 1
	203079_s_at	CUL2	Cullin 2
	203078_at	CUL2	Cullin 2
	203074_at	ANXA8	Annexin A8
	202867_s_at	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12
	202866_at	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12
	202865_at	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12
	202777_at	SHOC2	soc-2 suppressor of clear homolog (C. elegans)
	202731_at	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
	202730_s_at	PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
	202524_s_at	SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2
	202523_s_at	SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2
	202432_at	PPP3CB	Protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)
	202364_at	MXI1	MAX interacting protein 1
	202361_at	SEC24C	SEC24 related gene family, member C (S. cerevisiae)
	201859_at	PRG1	Proteoglycan 1, secretory granule

TABLE 2: Continued.

LOH gene clusters	Gene name	Common	Description
	201858_s_at	PRG1	Proteoglycan 1, secretory granule
	201753_s_at	ADD3	Adducin 3 (gamma)
	201752_s_at	ADD3	Adducin 3 (gamma)
	201376_s_at	HNRPF	Heterogeneous nuclear ribonucleoprotein F
	201366_at	ANXA7	Annexin A7
	201034_at	ADD3	Adducin 3 (gamma)
	200931_s_at	VCL	Vinculin
	200930_s_at	VCL	Vinculin
	200871_s_at	PSAP	Prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)
	200866_s_at	PSAP	Prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy)
	200697_at	HK1	Hexokinase 1
13q11q12	36830_at	MIPEP	Mitochondrial intermediate peptidase
	221995_s_at	MRP63	Mitochondrial ribosomal protein 63
	221771_s_at	HSMPP8	M-phase phosphoprotein, mpp8
	220753_s_at	CRYL1	Crystallin, lambda 1
	218479_s_at	XPO4	Exportin 4
	213262_at	SACS	Spastic ataxia of Charlevoix-Saguenay (sacsin)
	208742_s_at	SAP18	sin3-associated polypeptide, 18 kDa
	208741_at	SAP18	sin3-associated polypeptide, 18 kDa
	208740_at	SAP18	sin3-associated polypeptide, 18 kDa
	208590_x_at	GJA3	Gap junction protein, alpha 3, 46 kDa (connexin 46)
	207302_at	SGCG	Sarcoglycan, gamma (35 kDa dystrophin-associated glycoprotein)
	206404_at	FGF9	Fibroblast growth factor 9 (glia-activating factor)
	204387_x_at	MRP63	Mitochondrial ribosomal protein 63
	204386_s_at	MRP63	Mitochondrial ribosomal protein 63
	204305_at	MIPEP	Mitochondrial intermediate peptidase

was identified in SS cases by using Affymetrix 10 K Gene-Chip, which covers approximately 400 MS loci. There was an overlap between this and previous studies on LOH at chromosome 1p (D1S247) and 10q (D10S562). Previous studies also suggested LOH at 1p and 10q associated with late stage CTCL [15–17, 19, 48]. In addition, copy number losses were described in these 9 chromosomal regions by using molecular cytogenetic techniques as discussed above. Furthermore, these findings were subsequently validated by extensive SNP qPCR test in this study. Taking together all of these results suggest that some of these SNP LOH and copy number changes are likely to be associated with the pathogenesis of SS.

The FAT gene, which is expressed at high levels in a number of foetal epithelia, is an ortholog of the Drosophila fat gene. FAT encodes a tumour suppressor essential for controlling cell proliferation during Drosophila development. Its product is a member of the cadherin superfamily, a group of integral membrane proteins characterised by the presence of 34 tandem cadherin-type repeats as well as five epidermal growth factor-like repeats and one laminin A-G domain with possible functions as an adhesion molecule and/or signalling receptor, which are likely to be associated with developmental processes and cell communication (http://www.ncbi.nlm.nih.gov/gene/2195). The FAT gene is located on human chromosome 4q35. Previous molecular cytogenetic studies have revealed copy number losses at this chromosomal region in a variety of cancers (http://www.progenetix.org/cgi-bin/pgCasesMatrix-Plotter.cgi). Furthermore deletion/LOH and absent expression of FAT have also been found in oral cancer [53] and human astrocytic tumours [54]. Intriguingly, in this study SNP copy number loss of FAT was observed in three-quarter of SS cases and reduced gene expression of FAT was also noted in most cutaneous squamous cell carcinoma cases (data not shown) no spare SS RNA samples are available for qPCR testing. These results further indicate the tumour suppressor role of FAT and also suggest that loss of FAT is relevant to SS pathology.

Interindividual copy number variation/polymorphism (CNV/P) is thought to be the manifestation of a considerable and unanticipated plasticity of the human genome. CNV/P constitutes a major source of interindividual genetic variation that could explain variable penetrance of inherited diseases and variation in the phenotypic expression of aneuploidies and sporadic traits [55, 56]. There is increasing

evidence showing de novo CNV/P as a major cause of mental retardation and several other complex disorders [38–40, 43, 57]. Cytogenetically CNV/P or copy number changes (CNCs) have long been described in cancer cells (http://www.cgap.nci.nih.gov/Chromosomes/Mitelman, http://www.progenetix.net/). As discussed above SS has been found with frequent CNCs at several chromosomal regions [6–13]. Some of these CNCs may have a net effect on gene expression as previous expression profiling studies on SS have revealed up- and downregulations of several genes, which are mapped within the chromosomal regions harbouring these CNCs [20–26]. From, bioinformatic point of view these CNCs may be functional. Whilst the remaining CNCs (CNV/P) may not have any net effect at all and are therefore nonfunctional. To test this hypothesis we developed a simple bioinformatics strategy through the analysis of the expression profiling of CNCs gene clusters commonly presented in SS to see if SS cases can be separated from NCs. We found separated expression patterns in 4 of 17 CNCs gene clusters [46]. In this study, we expanded the scope of our bioinformatics experiment by examining the impact of common SNP LOH gene clusters identified in SS cases with Affymetrix 10 K SNP microarray on gene expression patterns in SS cases detected using gene expression microarray with Affymetrix GeneChip U133A and dedicated software. There was a separation of all 6 SS cases from 2 NCs by 3 of 9 SNP LOH gene clusters. This includes 9q31q34, 10p11q26, and 13q11q12, which lie within the chromosomal regions showing frequent LOH and/or DNA copy number loss in SS. In addition, decreased expression of MXI1 in SNP LOH gene cluster at 10p11q26 has been described in SS previously [22]. All of these findings suggest that the 3 SNP LOH gene clusters may be functional in terms of bioinformatics and relevant to SS pathology. There are also a number of genes in these 3 SNP LOH gene lists which have not been described in SS before. Further functional studies, including proteomic analysis, are therefore necessary to elucidate the oncogenic role of these genes in the initiation and progression of SS and other types of CTCL.

Acknowledgments

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Review Article

The Role of Dicentric Chromosome Formation and Secondary Centromere Deletion in the Evolution of Myeloid Malignancy

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Dicentric chromosomes have been identified as instigators of the genome instability associated with cancer, but this instability is often resolved by one of a number of different secondary events. These include centromere inactivation, inversion, and intercentromeric deletion. Deletion or excision of one of the centromeres may be a significant occurrence in myeloid malignancy and other malignancies but has not previously been widely recognized, and our reports are the first describing centromere deletion in cancer cells. We review what is known about dicentric chromosomes and the mechanisms by which they can undergo stabilization in both constitutional and cancer genomes. The failure to identify centromere deletion in cancer cells until recently can be partly explained by the standard approaches to routine diagnostic cancer genome analysis, which do not identify centromeres in the context of chromosome organization. This hitherto hidden group of primary dicentric, secondary monocentric chromosomes, together with other unrecognized dicentric chromosomes, points to a greater role for dicentric chromosomes in cancer initiation and progression than is generally acknowledged. We present a model that predicts and explains a significant role for dicentric chromosomes in the formation of unbalanced translocations in malignancy.

1. Introduction

Dicentric chromosomes, which have two centromeres, are a well-known feature of cancer cells, and the genome instability and evolution they induce are highly relevant to cancer biology [1, 2]. Although constitutional dicentric chromosomes are much rarer, in those that have been identified there is little evidence of this instability. Our studies have shown that the mechanisms by which dicentric chromosomes are stabilized include the loss of a centromere from a dicentric chromosome making it secondarily monocentric [3, 4], a previously little known mechanism which may be relatively common in cancer evolution. We review evidence that dicentric chromosomes have a greater role in oncogenesis than is currently acknowledged.

During cell division the two centromeres of an unstable dicentric chromosome migrate towards opposite poles at anaphase, causing cycles of breakage and rejoining which create new chromosome arrangements, deletions, and amplifica-tions. This is known as the bridge-fusion-breakage (BFB) cycle [5]. The gene copy number aberrations which are a byproduct of dicentric chromosome instability can create an increased risk of malignancy. Positive selection of these copy number changes can drive clonal evolution [6–10].

Copy number aberration (CNA) in the genome—net gain or loss of genetic material—is seen as one of the major causes of cancer. CNA can occur by whole chromosome gain or loss (aneuploidy), simple deletion or duplication of a chromosome segment, or unbalanced translocation where rearrangement between two or more chromosomes produces net gain or loss of material. Isodicentric chromosome formation causes both gain and loss of material, by the joining together of sister chromatids at a double-strand break, and is one method by which unstable dicentric chromosomes can be produced [11, 12].

Dicentric chromosomes in constitutional (nonmalignant) genomes are typically either stable at formation, or have

undergone a process of stabilization by mechanisms which have been well studied [13–16]. The processes underlying the stabilization of dicentric chromosomes in cancer cells have not been so well defined.

We will compare patterns of dicentric chromosome stabilization in the cancer and constitutional settings, including insights gained from our studies of cases of acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) with unbalanced karyotypes.

2. Dicentric Chromosomes in Malignancy

Dicentric chromosomes in cancer are best known as the product of telomere fusion events. Telomeres cap and stabilize the ends of chromosomes. Loss of this protective function, such as by a gradual reduction in the number of DNA telomere-specific repeats as a consequence of repeated genome replication, creates sticky chromosome ends. These can join to each other, with or without complete loss of the residual telomere and subtelomere sequences [17, 18]. Apparent telomere fusion (telomere association) events are characteristic of certain tumours, such as giant cell tumor of the bone [19] and meningioma [20]. Several model systems have been developed for creating and studying dicentric chromosomes *in vitro* or in nonhuman organisms, by artificially induced telomere erosion [7, 10, 18, 21–23]. Recent studies have reported direct molecular evidence for naturally occurring telomere fusion in cancer cells [6, 24].

Studies of dicentric chromosomes *in vivo*, *in vitro*, and in mice with induced telomere dysfunction, support a major role for telomere erosion and subsequent end-to-end chromosome fusion in causing the genome instability which is observed in many types of cancer [6, 8, 21, 22, 24–26], although this has also recently been questioned [27]. The BFB cycle undergone by these dicentric chromosomes is understood to be one of the principle causes of genome instability [11, 26, 28].

Unstable dicentric chromosomes can produce rearrangements such as deletion, amplification, inversion, double minute, and ring chromosomes [9, 28, 29]. Micronuclei and other nuclear anomalies can be produced as byproducts of instability. These contain centric or acentric chromosome segments which have not segregated to either daughter cell [26, 30]. Lagging of dicentric chromosomes so that they are then lost to a micronucleus, or missegregation of the whole dicentric chromosome to one daughter cell, can produce whole chromosome aneuploidy [21, 26].

Studies of transformed cell lines and premalignant cells have shown that with continued cell division and shortening of the telomeres, the cells enter a period of crisis, which is associated with end-to-end fusion of the chromosomes and genome instability, causing genome aberration. These cells with aberrant genomes would normally undergo senescence, but can survive if p53-induced apoptosis is inactivated. Telomere length and stability are restored by upregulation of telomerase or the alternative lengthening of telomere (ALT) mechanism [6, 8, 31–34].

Translocation between two or more chromosomes with interstitial breakpoints (reciprocal translocation) can also
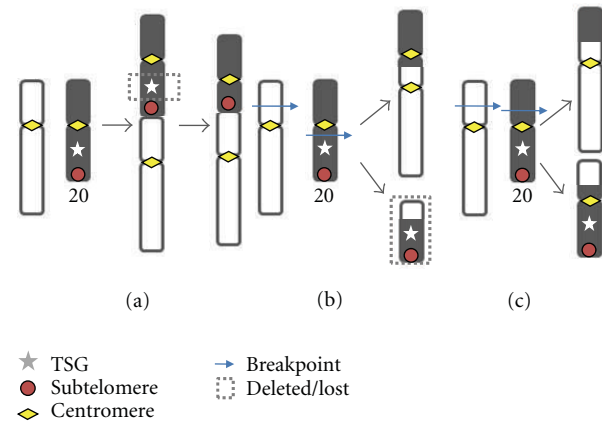

Figure 1: Translocations described in the text. (a) *Telomere Fusion*, creating a dicentric chromosome with minimal DNA loss but potentially causing secondary copy number aberration via the BFB cycle. (b) *Dicentric Translocation*, a reciprocal translocation producing a dicentric chromosome, and an acentric chromosome which is lost at cell division; (c) *Balanced Reciprocal Translocation*, producing two monocentric chromosomes—another event is required for CNA to occur. Our model proposes that (a) and (b), which both produce a dicentric chromosome, are the major causes of unbalanced translocation.

produce dicentric chromosomes (Figure 1). Hematological malignancies have the most well characterized chromosome abnormalities of any malignancy, and most dicentric chromosomes reported in hematological malignancies have a morphology consistent with this type of rearrangement rather than telomere fusion. Morphology can be misleading however, and there is now evidence that over half of the dicentric chromosomes involving chromosome 20 in myeloid malignancy are formed by telomere fusion events [35]. It is not yet known whether this pattern extends to other chromosomes.

Dicentric chromosomes which have been identified in hematological malignancies include the recurrent dic(17;20) [36, 37] and dic(5;17) [38] in MDS and AML and the dic(9;20) in acute lymphoblastic leukemia [39], as well as a range of other abnormalities [3, 39–41]. These are usually interpreted as unbalanced reciprocal translocations (see Figure 1), with breakpoints often described at or near the centromere [40, 42, 43]. The dic(9;20) is an unusual case. While most dicentric chromosomes, including the recurrent dic(17;20) [4], appear to have a range of breakpoints on both chromosomes, the dic(9;20) has been shown to have breakpoints within a single gene on 9p, *PAX5*, creating a fusion gene between this gene and a number of different genes on 20q [44–47].

Studies which focus on the identification of dicentric chromosomes in hematological malignancies have tended to uncover a higher incidence of dicentric chromosomes than is usually reported. In one of the rare studies into the incidence of dicentric chromosomes in myeloid malignancy, Andersen and Pedersen-Bjergaard [42] identified a dicentric chromosome in 15% (27/180) of consecutive cases of therapy-related AML (t-AML) and t-MDS and a much lower incidence in
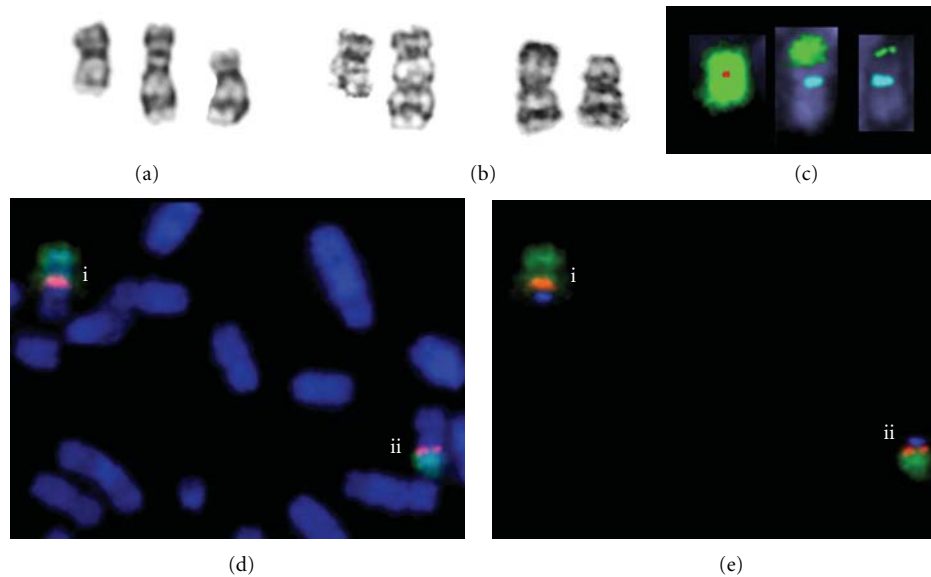
(a)

(b)

(c)

(d)

(e)

FIGURE 2: (a-b) G-banded chromosomes. (a) A typical stable dic(17;20) (centre) with the normal 20 (left) and 17 (right) from the same metaphase (Case 3 of Patsouris et al. [36]). (b–d) examples from a case with a highly variable dic(17;20) (Case 6 of MacKinnon et al. [4], SVH01 of MacKinnon et al. [48]). (b) Left, normal chromosome 20 and unstable dic(17;20) from a single metaphase. Right, two secondarily monocentric chromosomes with a 17 centromere (der(17)) from the same patient—each was derived from the primary dic(17;20) translocation by rearrangements which included loss of the 20 centromere. Both show a monocentric morphology and neither is easily recognizable as a pseudodicentric chromosome. (c) FISH images of the normal chromosome 20 and the two der(17)s illustrated in (b) (from a single metaphase). False colour images after FISH show the 20 centromere (red) (missing from the der(17)s), 17 centromere (aqua) (present in the der(17)s), and chromosome 20 content (green). (d-e) Two dic(17;20)s from the case illustrated in (b) and (c), both of which have secondary rearrangements bringing the centromeres closer together. (d) Blue, chromosomes stained with DAPI; green, whole chromosome 20 paint; red 20 centromere; (e) Blue, 17 centromere; green, whole chromosome 20 paint; red, 20 centromere. The derivatives shown have (i) inversion of chromosome 20 material and (ii) deletion of intercentromeric material.

de novo disease (0.4%). The combined incidence for AML and MDS was 8%. Callet-Bauchu et al. [41] identified a high incidence of dicentric chromosomes (10/14) among 17p translocations in chronic B-lymphoid disorders.

3. Dicentric Chromosomes in Myeloid Malignancy

A diverse range of cytogenetic aberrations has been described in the myeloid malignancies AML and MDS, including highly specific balanced translocations producing fusion genes, and copy number aberrations. Our studies of *in vivo* karyotype abnormalities in patients with these diseases have revealed a high incidence of dicentric chromosomes in unbalanced translocations involving chromosome 20.

We characterized the centromere and chromosome content and organization of 32 unbalanced chromosome 20 translocations, including thirteen unbalanced 17;20 translocations [3, 4, 36, 48]. These 32 cases had been identified as having apparent monosomy 20 (24 cases) and/or a 17;20 translocation (13 cases) (five cases fulfilled both criteria). Most had lost the putative tumor suppressor gene (TSG) region at 20q12.

Eight cases of dic(17;20) had a typical morphology comprised of 20p, 17q, and the proximal regions of 20q and 17p between the two centromeric constrictions [3, 36] (Figure 2(a)). A further five cases had a variant 17;20 trans-

location derived by secondary rearrangement of a primary dicentric chromosome [4, 48] (e.g., Figures 2(b)–2(e)). Thus, every one of thirteen 17;20 translocation products that we identified had been formed as a dicentric chromosome [4, 36, 48],

Twenty-one of 24 cases (87.5%) with apparent monosomy 20 (including the five variant 17;20 translocations) were shown to have a primary dicentric translocation, and only six of these appeared to have no secondary rearrangement or epigenetic inactivation of a centromere (Table 1) [3]. There was not enough information to determine whether the translocations of the remaining three cases had been derived from dicentric chromosomes.

These findings point to a hidden body of dicentric chromosomes in myeloid malignancy, that can only be identified by detailed characterization. In a review of dicentric chromosomes in hematological malignancies, Berger and Busson-Le Coniat [40] reported that the identification of dicentric chromosomes is increased when fluorescence *in situ* hybridization (FISH) with specific α-satellite DNA probes is used and suggested that the true incidence is higher than realized. Our studies show that even the use of centromere probes will not identify all dicentric translocations, because some primary dicentric chromosomes become secondarily monocentric. Our evidence points to a frequency of primary dicentric chromosomes in AML/MDS which is higher than the 8% reported by Andersen and Pedersen-Bjergaard [42] and is

TABLE 1: Patterns of secondary chromosome aberration derived from 32 primary dicentric (20;var) chromosomes in cases of AML and MDS described in previous publications. In total eleven cases had some mechanism of secondary centromere deletion and had retained or lost the deleted segment, and eight had another type of rearrangement which produced an altered (secondary) dicentric chromosome which retained both chromosomes (four cases had mixtures of clones exhibiting both mechanisms).

Secondary event	MacKinnon and Campbell [3]	MacKinnon et al. [4]	Patsouris et al. [36]	MacKinnon et al. [48]	Total
No change	6	4	1		11
Centromere inactivation	3				3
Intercentromeric deletion	1				1
Inversion reducing intercentromeric distance	3				3
Cases with secondary monocentric chromosomes:					
Mixture of clones: intercentromeric deletion/centromere deleted		1			1
Mixture of clones: intercentromeric deletion/centromere excised and retained in a ring chromosome	1				1
Centromere deleted	5				5
Centromere excised and retained in a ring chromosome	2				2
Complex mixture of clones: dicentric and secondary rearrangements (centromere deletion, excision of centric ring, inversion, deletion)		1		1	2
Total	21	6	1	1	29

potentially as high as the frequency of unbalanced translocations in AML/MDS. In our laboratory during 2009 and 2010, the frequency of karyotypes with unbalanced translocation in new cases of MDS or AML was 18%.

4. Stabilization of Dicentric Chromosomes in Myeloid Malignancy

These detailed studies of dicentric chromosomes in cases of AML and MDS gave us the opportunity to identify some of the mechanisms by which dicentric chromosomes had been stabilized. In 18/29 primary dicentric chromosomes, some or all of the cells had undergone secondary events which could be interpreted as producing a more stable derivative. A summary of these different secondary events is presented in Table 1. One case had a highly unstable dic(17;20) with 26 Mb between the centromeres, that had produced a wide range of derivatives (Figure 2) [4]. The variety of rearrangements is reminiscent of those described by Riboni et al. [2] in a dicentric chromosome which was produced by telomere fusion *in vitro*.

Centromere suppression occurred by functional inactivation (which was assumed when α-satellite DNA was still present but there was no centromeric constriction) or by excision of the α-satellite DNA. (Centromere suppression refers to loss of centromere function, regardless of how this is achieved). Loss of centromere-specific α-satellite DNA from the chromosome was the most common type of secondary rearrangement. The dicentric chromosome of 11 cases had become secondarily monocentric, by deletion of a centromere from a primary dicentric chromosome. In some cases the excised segment containing the centromere was retained

in the cell as what appeared to be a ring chromosome (Table 1).

We identified loss of the 20, 6, or 17 centromere in these cases [3, 4]. We have also identified cases where there has been loss of the 17 centromere from a primary dicentric chromosome not involving chromosome 20 (RNM, unpublished results; Figure 3). Similar studies will be needed to determine if centromere deletion occurs more widely in dicentrics involving other chromosomes and in other malignancies.

Some secondary rearrangements were produced by intercentromeric deletion or inversion of the inferred primary dicentric chromosome (Figures 2(d), 2(e)). These rearrangements produced dicentric derivatives with a greatly reduced intercentromeric distance, apparently resulting in stable dicentric chromosomes. The stable dicentric chromosomes showing no evidence of secondary events had short intercentromeric distances, supporting studies in constitutional dicentric chromosomes which show that this property renders the dicentric chromosome stable [14, 49].

More than half of the dic(20;var) which had lost the 20q12 TSG region had done so by an interstitial deletion, retaining the distal, subtelomeric 20q region [35]. Based on evidence that dicentric chromosomes can occur by telomere fusion, the simplest explanation of our findings was that an unstable primary dicentric chromosome had been formed by telomere fusion between chromosome 20 and another chromosome, followed by positive selection of a derivative with a secondary 20q12 deletion (see Figure 1). However, the alternative explanation, translocation between a preexisting del(20q) and another chromosome, could not be discounted.
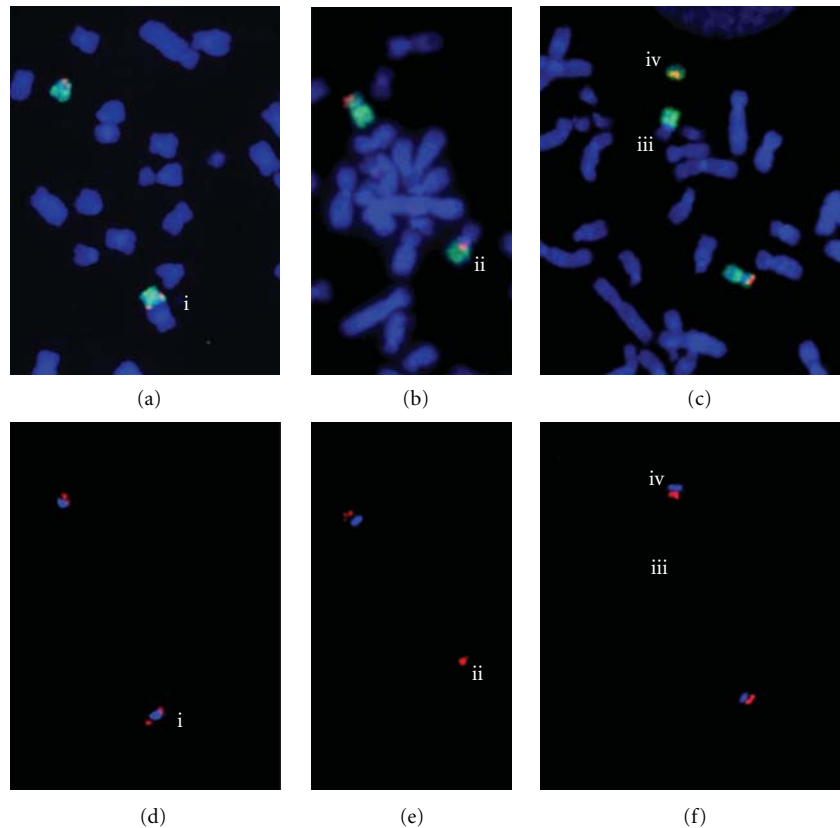
FIGURE 3: FISH images of three partial metaphases from a case with a dic(16;17) and derivatives formed by loss of the 17 centromere. The normal 17 is also present in each image. (a–c) Blue, chromosomes; red, *TP53* (a 17p13 locus); green, whole chromosome 17 paint; (d–f). Each image shows the same view as the image above it but the labelled 17 centromere is visible: blue, 17 centromere; red *TP53*. (a) (i) a dic(16;17)(q12.1;p13). (b-c) derivatives of the dic(16;17). (b) (ii) a der(16) (the 17 centromere has been lost). (c) (iii) a der(16) and (iv) the deleted segment containing the 17 centromere and *TP53*. Methods for FISH were as described in MacKinnon et al. [4].

We have described five cases of dic(17;20) with interstitial deletion of 20q suggestive of telomere fusion and subsequent deletion of the 20q12 TSG containing region [4, 35, 48]. A telomere fusion between 17p and 20q would produce a dicentric chromosome with an intercentromeric distance of 56 Mb. According to studies of constitutional dicentric chromosomes [14, 49] a dicentric chromosome with this intercentromeric distance would be unstable. Thus, the secondary deletion of 20q12 could fulfill two roles: loss of a TSG and stabilization of the dicentric chromosome.

We have also reported localized amplification of a section of 20q (20q11.21), in both MDS and AML, the development of which was probably aided by its position between the centromeres of unstable dicentric chromosomes [3, 48]. We have suggested that this selectively amplified region contains an oncogene [3, 48]. As well as deletion, the BFB cycle can cause amplification of material between the centromeres of a dicentric chromosome. Positive selective pressure would tend to favor amplification of an oncogene in this position. Gain of the whole abnormal chromosome in other cases, rather than localized amplification, provides further support for on oncogenic role for a gene in this region. Further instances of amplification of 20q11.21 have been identified since these publications (RNM, unpublished observations).

5. Centromere Deletion

Centromere deletion or excision has only rarely been described previously. Nevertheless, it was a significant event in our series (occurring in 11 of 29 primary dicentrics) and was more frequent than functional inactivation. FISH with BAC (bacterial artificial chromosome) clones was used to define the breakpoints of 20 centromere deletion [48], which fell into two main categories. Some had deletion breakpoints close to the centromere, retaining part or all of the flanking BACs (two published cases [48] and one unpublished case (RNM, unpublished results)), while others had excision of a broader section including 20q11.21, and the excised fragment was retained in the cell as either a ring or marker chromosome (4 cases). We have also observed larger deletions spanning the centromere without retention of the deleted segment (chromosome 17, one case and chromosome 20, two cases) [4, 48]. Some cases had multiple clones with different centromere deletion events. There was no FISH evidence of partial retention of the centromere in any of these cases.

In the 1970s it was suggested that centromere suppression could be a result of either deletion of the centromere or functional inactivation [50–52]. However, molecular studies since

then have identified epigenetic mechanisms for suppressing centromeres, that is, loss of the centromere proteins which form the kinetochore, produces a functionally inactive centromere [13, 53]. These studies were carried out on constitutional dicentric chromosomes, but it seems to have been generally assumed that functional inactivation is also the mechanism of centromere suppression in cancer cells (e.g., [40]). Although dicentric chromosomes are commonly found in cancer cells, mechanisms stabilizing these dicentric chromosomes have not been well studied.

In humans, centromere deletion from a dicentric chromosome has been noted most often in constitutional dicentric Y chromosomes [54–57], but also in a handful of other cases [58, 59]. The α-satellite DNA was found to be only partially removed in some of these, including the two examples of isodicentric Y chromosome which were studied in detail by Tyler-Smith et al. [54], a landmark study which used these deletions to identify α-satellite DNA as the sequence marking the centromere. In some cases [55, 56, 58, 59] the excised section, including the centromere, was preserved in a small stably transmitted marker chromosome. Rivera et al. [55, 56] identified at least six cases with pseudodicentric Y chromosomes in which the excised Y centromere was still present, in a small marker chromosome. These authors have suggested that centromere excision from pseudodicentric Y chromosomes with retention of the excised section is underdiagnosed, because these marker chromosomes can usually only be detected by FISH [55].

Centromere deletion has also been identified in yeast. Artificially created dicentric chromosomes in *Saccharomyces cerevisiae* can be stabilized by deleting a section of DNA containing one centromere [60, 61].

Stimpson et al. [7] observed partial α-satellite deletion in dicentric human chromosomes that were created *in vitro*, in fibrosarcoma cell lines. However, these dicentric chromosomes had been artificially induced by *in vitro* abrogation of telomere function.

Our studies identifying centromere deletion in MDS and AML combined FISH for specific centromeres with traditional cytogenetics, multicolor FISH (M-FISH), and multicolor banding (M-BAND) [3, 4, 48]. To our knowledge ours are the first studies identifying centromere deletion from naturally occurring cancer chromosomes. Centromere deletion or excision was the most common mechanism of dicentric chromosome stabilization that we identified in abnormalities of chromosome 20. This suggests that there is a significant rate of unidentified centromere deletion in cancer genomes.

6. Functional Inactivation of a Centromere

While dicentric chromosomes are relatively common in cancer cells, they are rare in the constitutional setting, probably because most events causing genome imbalance are incompatible with embryo viability. Nevertheless, most studies of *in vivo* dicentric chromosome stabilization in humans have been carried out on constitutional dicentric chromosomes discovered through clinical cytogenetic analysis.

Stabilization of these dicentric chromosomes has generally occurred by the time of discovery [13–16].

The most commonly reported constitutional dicentric chromosomes are Robertsonian translocations and isodicentric X chromosomes—which have lost nonessential material. Stability is typically achieved through close physical proximity of the centromeres [14, 49] or suppression of one of the centromeres [16, 62, 63].

In a series of isodicentric X chromosomes in patients with Turner syndrome, Sullivan and Willard [49] determined that an intercentromeric distance of 12 Mb or less is compatible with a stable, functionally dicentric chromosome (in which both centromeres are active), whereas a larger intercentromeric distance is not compatible with stability. Dicentrics with more material between the centromeres (at least 34 Mb in their examples and 15 Mb in a recent paper by Ewers et al. [64]) consistently had only one active centromere. Available evidence points to a requirement for rapid stabilization of a dicentric chromosome with well-separated centromeres, if the (nonmalignant) cells are to remain viable [49, 65, 66].

Centromere suppression in constitutional dicentric chromosomes with well-separated centromeres has most often been shown to be achieved by functional inactivation, producing a pseudodicentric chromosome. Functional inactivation occurs by loss of the centromere proteins which assemble at the kinetochore and define an active centromere [13, 53, 67–70]. Antibodies to some of these epigenetic markers, CENP-A, CENP-C, and CENP-E, have commonly been used to distinguish between functionally active and inactive centromeres [7, 13, 53]. Page and Shaffer [14] showed that the centromere-specific alpha satellite DNA of both centromeres was maintained in dicentrics with one inactive centromere.

Even in stable dicentric chromosomes with closely apposed centromeres, there is usually a mixture of cells with one or two active centromeres, as has been shown in Robertsonian translocations [13, 14], isodicentric X chromosomes [49, 71], and other constitutional dicentrics [50, 66]. The patterns of inactivation seen in parent-child pairs were consistent with stable transmission of the centromere in either the active or inactive state, with a gradual progression towards loss of functionality of either centromere if both were inherited in the active state [14]. In cultured cells there tended to be a higher proportion of functional monocentrics (structural dicentrics with one active centromere) [13, 14], consistent with a tendency to centromere inactivation but not reactivation.

It is not known whether centromere reactivation occurs *in vivo* in humans. The presence of CENP-A is usually a prerequisite to kinetochore assembly in humans [68, 72–74]. However, centromere DNA lacking centromere proteins can be reconstituted (reactivated) in yeast, maize, and mammalian artificial chromosomes *in vitro* [75–78]. Also, in rare instances a new human centromere (neocentromere) can be created at a position naïve of centromere α-satellite DNA by assembly of the necessary proteins, to preserve a marker chromosome that lacks a native centromere [79]. This suggests that reactivation of a human centromere *in vivo*

is possible, but there have been no conclusive reports of this occurring [14, 15, 59, 80, 81].

Stimpson et al. [7] looked at mechanisms of stabilization of artificially created dicentric chromosomes. The fate of the centromeres in these dicentric chromosomes was followed from the time the dicentric chromosomes were formed. There was progressive centromere suppression after several generations by functional inactivation and, less commonly, partial deletion of α-satellite DNA at the centromeres as noted above.

Stabilization of double-strand DNA breaks in dicentric chromosomes by the addition of new telomeres has been described in mouse embryonic stem cells and tumor cells [17, 82, 83]. Similarly, splitting of a dicentric chromosome into two monocentric derivatives has been described in humans [2] and yeast [84].

7. Identification of Dicentric Chromosomes and Secondary Rearrangements

A number of factors can explain why secondary centromere deletion in malignancy had not been recognized earlier. These include the tendency to explain pseudodicentric chromosomes as having been derived by centromere inactivation and the infrequency of testing for the presence of centromeres in routine metaphase cytogenetic analysis. In our experience, monocentric chromosomes derived from dicentric chromosomes were often only identified after a detailed analysis of centromere content, chromosome content, and organization.

FISH studies to detect pairs of centromeres are not usually carried out on metaphase chromosomes during diagnostic cancer cytogenetic analysis. The chromosomes that are most likely to be recognized as dicentric are those stable primary dicentric chromosomes that have two distinct centromeric constrictions or a recognizable pseudodicentric morphology spanning both centromeres. Other secondary monocentric chromosomes are morphologically unrecognizable because of the altered morphology around the centromere, as is the case with chromosome 20 morphology after the centromere has been deleted [3, 85] (Figure 2(b)). Furthermore, dicentric chromosomes that break into two monocentric derivatives might not be identified as having been derived from a primary dicentric chromosome.

The use of array karyotyping (single nucleotide polymorphism—SNP—and comparative genomic hybridization—CGH—arrays) on its own for cytogenetic analysis will not identify dicentric chromosomes, telomere fusion, or centromere deletion. Centromeres and telomeres are not represented on standard microarrays, nor do microarrays give information on translocation partners or chromosome organization. As residual telomere sequence can be retained at the site of telomere fusion [2, 6, 86], there will not necessarily be loss of the distal part of the chromosome that is detectable by microarrays, in dicentric chromosomes formed by telomere fusion. In the two cases of apparent telomere fusion that we analyzed by array CGH [48], there was no detectable terminal deletion of 20qter, nor did the arrays show any indication of the centromere loss that had occurred. This

may have clinical implications. Dicentric chromosomes may have a poorer prognosis because they can be subject to more rapid selective evolution.

If on any derivative chromosome both translocation partners contain material spanning the centromeres, this is the strongest indication that there was a primary dicentric translocation. A method for identifying chromosome content (array karyotyping, M-BAND, or traditional banding) in conjunction with FISH for the centromeres (using a pancentromere probe or specific centromere probes or, more efficiently, multicolor centromere FISH (cenM-FISH) which will identify all the centromeres in one step [87]) and a method for identifying translocation partners (M-FISH or traditional banding) can therefore help identify dicentric chromosomes, including primary dicentric chromosomes which have lost one of the centromeres. M-BAND [88] gives information on gross chromosome content and organization within an individual cell and also helps define different clones. It can help shed light on the rearrangements that have occurred, if this information is not provided by standard karyotyping. It may not be practical to carry out this level of analysis in routine diagnostics, but for a full understanding of the causes and consequences of unbalanced translocation in a research context, it may be essential.

8. The Tip of the Iceberg?

Our studies highlight a higher incidence of primary dicentric chromosome formation in unbalanced chromosome 20 abnormalities than has previously been recognized. The dic(20;var) appears to be an example of an unstable dicentric chromosome creating selectable derivatives that can allow rapid clonal evolution.

Our studies suggested a greater role for telomere fusion in creating dicentric chromosomes in myeloid malignancy than has been previously acknowledged. Environmental and biological factors associated with telomere erosion, that is, advancing age and chemical exposure, are also risk factors for developing MDS and AML [43, 89–91], and so identifying a high incidence of telomere fusion as an oncogenic event may help identify the causes of these diseases. Complex karyotypes often include many unbalanced translocations, frequently of unknown composition, and the resulting chromosomes are typically assumed to be monocentric unless there is clear evidence to the contrary. Given the apparently high incidence of unidentified chromosome 20 dicentrics in myeloid malignancy, we suggest that other unbalanced translocations in AML, MDS, and other malignancies may also harbor a higher incidence of dicentric chromosomes than is currently recognized. This would suggest that the instability associated with dicentric chromosomes is a much more significant factor in cancer than is apparent. We propose a model that both explains the high incidence of dicentric chromosomes in unbalanced chromosome 20 translocations and makes the more general prediction that dicentric chromosome formation is a major mechanism for unbalanced chromosome translocation in malignancy.

9. Model for the Formation of Unbalanced Translocations by Dicentric Translocation

This model suggests that most unbalanced translocations are formed by one of two mechanisms producing a dicentric chromosome, *dicentric translocation*, and *telomere fusion*. Genome imbalance is a direct consequence of both of these events. Three types of translocation are defined.

(i) *Telomere Fusion* (end-to-end joining of chromosomes at the telomeres): this is a balanced rearrangement (apart from possible telomere and subtelomere loss), but if the centromeres are well separated, the chromosome is unstable and further rearrangements are likely—including oncogenic deletions and amplifications—creating an unbalanced translocation product.

(ii) *Dicentric Translocation*: a simple reciprocal translocation producing a dicentric and an acentric chromosome. As the acentric chromosome is lost during mitosis, this becomes an unbalanced translocation.

(iii) *Balanced Reciprocal Translocation*: a simple reciprocal translocation producing two monocentric chromosomes. There is no net gain or loss of material and another event would be required to produce copy number aberration. Therefore we suggest that this is not a usual cause of genome imbalance, contrary to the tacit assumption that the products of most oncogenic unbalanced translocations are monocentric.

The novel aspect of this model is that it predicts that dicentric chromosomes play a much greater role in oncogenesis than is currently appreciated and gives a smaller role to balanced reciprocal translocation in the generation of copy number imbalance. A significant role for dicentric chromosomes produced by telomere attrition in causing genome instability is already recognized. Our chromosome 20 studies provide evidence for both mechanisms of dicentric chromosome formation and support only a minor or coincidental role for reciprocal translocation producing two monocentric chromosomes.

10. Conclusions

Dicentric chromosomes are rarely identified in constitutional genetics. However, in cancer cells the formation of dicentric chromosomes is a well-recognized event, which may contribute to the malignant phenotype and clonal evolution. We have explored the patterns of stabilization of dicentric chromosomes in constitutional karyotypes, cancer cells, and cells in tissue culture.

Our studies have shed a light on the role of dicentric chromosome formation in myeloid malignancy. Many primary dicentric chromosomes escape detection when traditional or molecular karyotyping is used to characterize genome aberrations. We have uncovered a significant role for centromere deletion or excision in the evolution of myeloid malignancy, which raises the possibility that this occurs

more widely, and in other cancers. More detailed studies combining molecular karyotyping (which allows precise breakpoint definition and genome-wide detection of copy number aberration) with FISH studies (which allow chromosome organization and centromere content to be determined) will lead to a better understanding of the role of dicentric translocations in cancer.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

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Review Article

Genetic Markers Used for Risk Stratification in Multiple Myeloma

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While no specific genetic markers are required in the diagnosis of multiple myeloma (MM), multiple genetic abnormalities and gene signatures are used in disease prognostication and risk stratification. This is particularly important for the adequate identification of the high-risk MM group, which does not benefit from any of the current therapies, and novel approaches need to be proposed. Fluorescence in situ hybridization (FISH) has been employed for establishing risk-based stratification and still remains the most used genetic technique in the clinical routine. The incorporation of gene expression profiling (GEP) in the study of MM has shown to be a very powerful test in the patient stratification, but its incorporation in clinical routine depends on some technical and logistic resolutions. Thus, FISH still remains the gold standard test for detecting genomic abnormalities and outcome discrimination in MM.

1. Background

Multiple Myeloma (MM) is a malignancy characterized by accumulation of clonal antibody-secreting plasma cells [1]. While no specific genetic markers are used for MM diagnosis, multiple genetic abnormalities have been associated with malignant transformation and disease progression [2–5]. The identification of genetics aberrations was greatly improved after the implementation of analytic tools capable to overcome the technical limitations related to low proliferation of the myeloma cell. Thus, several classifications have been proposed based on the identification of the genomic changes that help to discriminate between different genetic groups of MM patients [3, 6–9].

Overall, MM is divided into two main genetic groups: (1) the hyperdidploid group (H-MM), which can be defined mainly by the gain of odd chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 and (2) the nonhyperdiploid group (NH-MM), characterized by the presence of chromosomal translocations involving the immunoglobulin H *(IgH)* locus with several chromosomal partners (4, 8, 11, 16) [10–12]. Each category includes approximately half of cases, with a very low number of overlapping cases.

Of interest, the dissection of the genetic landscape has provided important genetic markers with demonstrated clinical and disease stratification value [5, 13–15].

2. Cytogenetic Prognostic Markers—FISH

2.1. t(4;14)(p16;q32). This translocation affects the telomeric portion of chromosome 4p leading to the dysregulation of two protooncogenes, *FGFR3* in derivate chromosome 14 (der14) and multiple myeloma SET domain *(MMSET)* in derivative chromosome 4 (der4) [16]. The t(4;14) is seen in 15–20% of primary MM [17]. The translocation is cryptic and detectable only by FISH or reverse transcriptase—PCR [17].

Several groups have associated the t(4;14) with inferior outcome and more aggressive disease irrespective of the treatment modality [2, 5, 18, 19] (Table 1). It has been suggested that this group of patients can benefit from bortezomib-based therapy [20]. However, two recent studies showed that, although bortezomib-based therapy shows better results than previous therapies (vincristine, adriamycin, and dexamethasone) in patients with t(4;14),

Table 1: Abnormalities associated with outcome in MM and techniques used for detection.

Abnormalities	Outcome	Test
t(4;14)(p16;q32)	Poor	FISH*
t(14;16)(q32;q23)	Poor	FISH
t(6;14)(p21;q32)	Good?	FISH
t(11;14)(q13;q32)	Good/neutral	FISH
Deletion 17p13	Poor	FISH
Deletion 13	Poor	Convenlional cytogenetics
Chromosome 1 abnormalities	Poor	FISH
Hyperdiploidy	Good	FISH or FCM**
(if not associated with deletion 17p13)		

*Fluorescence in situ hybridization (FISH). **Flow cytometry.

this translocation still has prognostic implications in a great group of patients treated with this drug [18, 19].

2.2. t(14;16)(q32;q23) and Other MAF Translocations. The t(14;16) is found in 5–7% of all MM cases [4, 5, 21]. The presence of t(14;16) has been associated with more aggressive disease and shorter survival among the patients treated with either conventional or high-dose chemotherapy [5, 6] (Table 1). The prognosis of this translocation was recently challenged by a study that suggests a neutral effect in a large series of patients [21]. Given the very low prevalence of the MAF abnormalities, the test to detect the presence of these translocations has not been universally incorporated in the clinical routine.

The upregulation of *CCND3* (cyclin D3), as a result of t(6;14)(p21;q32), is identified in only 3% of MM [5] (Table 1). Until now, there is no known clinical or prognostic information for this translocation.

2.3. t(11;14)(q13;q32). This translocation results in the juxtaposition of *CCND1* proto-oncogene with the *IgH* locus and as consequence an ectopic expression of cyclin D1 [22]. Of all MM, the *t(11;14)* has been described in 15% of cases and is associated with CD20 expression, lymphoplasmacytic morphology, hyposecretory disease, and Ig light chain usage [22, 23].

Most studies have suggested that the presence alone of t(11;14) may confer a favorable outcome (Table 1), but this effect is not strong enough to be statistically significant (probably because of small magnitude of this translocation) [22–24]. Moreover, due to heterogeneity within patients with t(11;14) there exists a difficulty in establishing a favorable outcome for patients with this genetic aberration. For instance, the presence of K-RAS mutations in patients with t(11;14) is also more prevalent (50%) than in patients with other primary IgH translocations (10%) [25]. In addition, the presence of t(11;14) is associated with an aggressive phenotype such as plasma cell leukemia [23]. A recent study with a larger series of patient with t(4;14) has suggested

that the effect of t(11;14) on prognosis remains neutral [24] (Table 1).

2.4. Ploidy Status. In MM, aneuploid is frequently observed [11, 12] and delineates the disease into two main genetic subtypes, H-MM and NH-MM. H-MM is more common among males, has a higher incidence of MM bone disease, and carries a more favorable outcome [6] (Table 1). Among patients with H-MM, 13 deletion and chromosome 1 abnormalities have not apparent prognostic significance but the presence of deletions of 17p13 in remains an important prognostic factor. In addition, a study showed that most of the prognostic value of H-MM was related to the gain of chromosome 5 [24, 26].

2.5. Deletion of 17p. The deletion of 17p13 remains the most important molecular prognostic factor in MM [5, 6, 20]. The deletion 17p13 is generally monoallelic and includes *TP53*. The abnormality is detected in only 10% of new diagnosis MM cases, but its prevalence increases in later stages of the disease. Patients with 17p13 deletions often have more aggressive and extramedullary disease (such as plasmacytomas), center nervous system involvement, and hypercalcemia [6, 27]. This abnormality is associated with a shorter survival irrespective of the treatment modality, including the novel bortezomib and IMiDs-based therapies [5, 6, 14, 27] (Table 1).

2.6. Chromosomes 1 and 13. Chromosome 1 abnormalities are found in almost half of MM cases [28]. There is an enrichment of genes associated with proliferation in the affected region [8]. Although the poor prognosis value of this abnormality has been recently demonstrated, its incorporation into standard clinical practice has not been implemented yet [28] (Table 1).

The deletion of chromosome 13 is found in 50% of MM cases [4–6, 8, 29]. Although this abnormality was originally identified as negative prognostic factor in MM, several studies had proved the association of chromosome 13 monosomy with the t(4;14) [6, 30, 31]. Even in the absence of this association or other high-risk markers, the chromosome 13 alone is not a predictive of poor prognosis when identified by FISH (Table 1). On the other hand, its identification by conventional cytogenetics is a surrogate of high proliferation and is used as a poor prognostic marker [32] (Table 1).

3. Comprehensive Genomic Tools in MM Risk Stratification

The advent of high-resolution genomics tools provided a remarkable revolution in the analysis of MM and the identification of genomic signatures able to identify high-risk patients and to predict patient outcome [6, 8, 33]. Several high-resolution available tests provide a comprehensive analysis at the DNA (aCGH, single-nucleotide polymorphism (SNP) arrays, and whole-genome sequencing (WGS)) and RNA levels (gene expression profiling (GEP)).

Among these technologies, the use of GEP is the most promising risk stratification tool in MM. The use of GEP has been successfully implemented in MM, and several genetic signatures have been proposed [8, 33, 34]. The most used signatures are based on the analysis of proliferation markers or in centrosome index and successfully detected the 15–20% of worse prognosis patients [35].

The prognostic classification, using genetic analysis as outcome discrimination, has been used in several cohorts of MM treated with the conventional and high-dose chemotherapy followed by stem cell transplant (SCT) [5, 8, 35]. Moreover, the ongoing studies involving patients with MM are focused on the use of these genetic markers provided by genetic changes, as predictors of outcome in those treated with proteasome inhibitors. Although GEP is still mainly used for research purposes, some groups have successfully implemented its use in the routine clinical care [35]. Other approaches such as aCGH and WGS have not been implemented in the clinical routine yet, being used in the research laboratory.

4. Conclusion

Genetic studies have played a crucial role in the determination of the risk-based stratification of MM. Nowadays, FISH and GEP are the most powerful tools for successfully identifying disease subgroups with different outcomes.

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Review Article

Molecular Alterations in Sporadic Primary Hyperparathyroidism

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Primary hyperparathyroidism (PHPT) is a frequent endocrine disorder characterized by an excessive autonomous production and release of parathyroid hormone (PTH) by the parathyroid glands. This endocrinopathy may result from the development of a benign lesion (adenoma or hyperplasia) or from a carcinoma. Most of the PHPT cases occur sporadically; however, approximately 10% of the patients present a familial form of the disease. The molecular mechanisms underlying the pathogenesis of sporadic PHPT are incompletely understood, even though somatic alterations in *MEN1* gene and CCND1 protein overexpression are frequently observed. The *MEN1* gene is mutated in about 30% of the parathyroid tumours and the protooncogene *CCND1* is implicated in parathyroid neoplasia by rearrangements, leading to an overexpression of CCND1 protein in parathyroid cells. The aim of this work is to briefly update the molecular alterations underlying sporadic primary hyperparathyroidism.

1. Introduction

One of the multiple implications of multicellularity is that all parts of a body must be able to communicate with each other, in order to maintain homeostasis. The communication between different parts of an organism is essential for an appropriate response to internal and external environmental stimuli. The endocrine system, through the production, and release of hormones, is a key element for the establishment and maintenance of such regulation [1].

The parathyroid glands are the endocrine organs responsible for regulating calcium levels by producing a hormone, the parathyroid hormone (PTH), released directly into the blood [2] that regulates calcium levels acting at various organs.

Calcium plays a fundamental role in controlling the neuromuscular activity, the blood clotting process, bone structure, and integrity of cell signaling. The levels of ionized calcium in bloodstream are maintained by a complex hormonal mechanism, involving three main systems: gastrointestinal system, bone, and kidney [3, 4].

Primary hyperparathyroidism is a common endocrinopathy characterized by an inappropriate oversecretion of PTH. High PTH levels lead to a hypercalcemic state affecting different organs and systems such as bones and kidneys and cardiovascular, gastrointestinal, and nervous systems [5, 6].

The incidence of PHPT is estimated as 25 cases per 100 000 individuals, and the main risk factors are older age, female gender, and exposure to cervical irradiation [7, 8].

This pathological condition, in 80 to 85% of the cases, is due to a parathyroid adenoma (uniglandular disease), followed by 15 to 20% of cases arising due to hyperplasia (multiglandular disease) and rarely, in 2 to 5% of cases, can be caused by a carcinoma [4].

Most of the PHPT cases belong to sporadic forms of the pathology, but about 10% of the cases are familial forms [7].

Germline genetic alterations in *MEN1* (*multiple endocrine neoplasia type 1*) gene are associated with PHPT in the context of MEN1 syndrome, and mutations in *HRPT2* (*hyperparathyroidism 2*) gene are associated with HPT-JT (Hyperparathyroidism-Jaw tumor) familial syndrome. The development of PHPT in the context of MEN2 (multiple

Table 1: Summary of the molecular alterations associated with familial and sporadic parathyroid tumors.

	MEN1 [20, 50–52]	CCND1 [31, 53–55]	HRPT2 [13, 56–58]	RET [48]	CaSR [49, 59–61]
Germline mutations (Familial syndrome)	Inactivating mutations, LOH (MEN1)	—	Inactivating mutations, LOH (HPT-JT)	Activating mutations (MEN2A)	Inactivating mutations (NSHPT/FHH)
Benign					
Somatic mutations	Inactivating mutations, LOH	Activating Inv (11) (p15; q13)	Inactivating mutations	N	N
Prevalence of somatic alteration	20 to 30%	~5%	2 to 4%	—	—
Protein expression (%)	Downregulation (20 to 40%)	Overexpression (30 to 40%)	Downregulation (ND)	ND	Downregulation (Up to 90%)
Malignant					
Somatic mutations	Inactivating	ND	Inactivating mutations, LOH	N	ND
Prevalence of somatic alteration	~13%	ND	70 to 100%	—	—
Protein expression (%)	ND	Overexpression (~90%)	Downregulation/loss of expression (70 to 100%)	ND	Downregulation (~30%)

N: negative; ND: not determined.

endocrine neoplasia type 2) syndrome, specifically in the variant MEN2A, is associated with genetic alterations in the *RET* (*rearranged during transfection*) gene. Homozygous or heterozygous mutations in *CaSR* (*Calcium Sensing Receptor*) gene are the source of NSHPT (neonatal severe hyperparathyroidism) and FHH (familial hypercalcemic hypercalciuric), respectively [9, 10].

Although, the genetic alterations associated with familial forms are well known, the genetic alterations underlying sporadic forms are far from being understood. The molecular alterations that are established as being characteristic of sporadic benign parathyroid tumor are genetic alterations in the *MEN1* gene (20 to 30% cases) and Cyclin D1 protein overexpression (30 to 40% of cases) [11, 12]. Parathyroid carcinomas are strongly associated with somatic *HRPT2* mutations [13].

1.1. The Role of MEN1 Gene in PHPT.

The *MEN1* tumor suppressor gene was identified in 1997 as the gene responsible for the autosomal dominant syndrome characterized by tumors of endocrine pancreas, the anterior pituitary and parathyroid glands, the MEN1 syndrome [14]. Apart from being involved in the mentioned familial syndrome, somatic mutations of *MEN1* gene are also implicated in the development of sporadic parathyroid tumors [15].

The *MEN1* gene is located on chromosome 11 (band 11q13), consists of 10 exons (with 9 coding exons), and encodes a protein of 610 amino acids called menin that is ubiquitously expressed, at all stages of development [16]. The transcript of *MEN1* gene is a 2.8 Kb mRNA but six more alternative transcripts have been reported, although none of them interferes with the coding region revealing variations of the 5′ translated region only [17]. Despite the high degree of

conservation among metazoan, menin does not reveal motifs of known function and has no similarity with any other known protein [18].

Tumors of MEN1 patients usually reveal the presence of a germline mutation followed by a somatic alteration such as loss of heterozygosity (LOH) or inactivating mutation, as predicted by the model of Knudson, pointing to *MEN1* gene as a very good example of a classical tumor suppressor gene [19].

About 30% of sporadic parathyroid tumors show *MEN1* gene mutations (see Table 1). These somatic mutations, similarly to what happen with the germline mutations, are spread throughout the coding region. About 40% of these mutations are frameshift, 29% are missense mutations, 18% are nonsense, 7% occur in splice sites, and 6% are deletions or insertions in-frame. Sporadic parathyroid tumors harboring *MEN1* gene somatic mutations frequently evidence LOH on chromosome region 11q13 [20]. Somatic inactivating mutations in this gene have also been identified in other types of tumors, namely, in neuroendocrine tumours such as gastrinomas, insulinomas, lung carcinoids, and pituitary tumors showing a similar loss of function mechanism promoting tumorigenesis [21–23].

From *Drosophila* to humans, menin is conserved but is absent in *C.elegans* and yeast [24]. Subcellular localization studies evidence that the protein has predominantly a nuclear localization and possesses two nuclear localization signals (NLSs) that should be essential for its role in regulation of gene transcription [25]. Menin has been reported to interact with a broad spectrum of proteins involved in regulation of transcription by coordinating chromatin remodeling, genome stability, cell division, and proliferation [26, 27].

Recently, it was demonstrated *in vitro* that menin acts as a scaffold and inhibits AKT/PKB activation by regulating its cellular localization, since it hampers the transition from the cytoplasm to the membrane, required to its activation [28].

The clinical relevance of pathways and molecular mechanisms in which menin is involved remain to be clarified.

Most of the *MEN1* gene mutations will lead to a premature stop codon, giving rise to a truncated protein. Frequently this truncated menin may have lost, at least one of its NLS, compromising the protein function, regarding their role of driving the protein to the nucleus, but also by coordinating the regulation of expression of menin target genes [25].

Menin protein can function as suppressor of transcription, because this protein is able to bind a family of transcription factors such as AP-1/Jun-Fos family, and it is also associated with a histone methyltransferase (HMT) complex leading to an increased expression of cyclin-dependent kinase inhibitors (CDKIs) and consequently suppressing cell growth [29, 30].

1.2. The Role of Cyclin D1 Protein in PHPT. The gene encoding cyclin D1, *CCND1*, located in chromosome 11q13, was initially designated *PRAD1* (*parathyroid adenomatosis 1*) since it was cloned from DNA from a parathyroid adenoma. In 1989, Arnold and colleagues found a genetic rearrangement in a parathyroid adenoma, this rearrangement, inv (11) (p15; q13), positions the 5′ PTH gene regulatory region (located in 11p15) adjacent to the *CCDN1* gene leading to the overexpression of cyclin D1 protein [31]. The increased expression of cyclin D1 promotes the transcription of genes required for DNA synthesis and cell cycle progression.

Several studies show the overexpression of cyclin D1 in 20–40% of parathyroid tumors (see Table 1), even though they do not demonstrate the presence of the rearrangement, pointing to the presence of other molecular abnormalities leading to an overexpression of this cell cycle regulator [32].

The pathogenic role of this protein was assessed through studies in transgenic mice overexpressing cyclin D1 in parathyroid. These mice were created to model the genetic rearrangement found in human parathyroid tumors [33]. In this model, the overexpression of this cell cycle regulator leads to hyperplasic parathyroid glands, with increased cell proliferation, retaining their capacity of hormone production [34].

1.3. Parathyroid Carcinoma and CDC73/HRPT2. In 2002, germline mutations in the tumor suppressor gene *hyperparathyroidism 2* (*HRPT2*) were described by Carpten et al. as being responsible for the HPT-JT familial syndrome [13].

HRPT2 gene is located at 1q25-q31 and encodes a 60 kDa nuclear protein named parafibromin that has been shown to be a member of the polymerase-associated factor (PAF1) complex involved in gene transcription regulation by histone ubiquitination and methylation [35, 36].

Additionally, parafibromin suppresses tumor growth by inducing apoptosis, inhibiting G1 to S phase transition, regulating Wnt canonical pathway, and also regulating gene expression by direct binding of promoter regions, being

therefore expected that loss of parafibromin will lead to enhanced cellular proliferation [37–40].

The loss of parafibromin as a molecular marker of parathyroid carcinoma was first reported by Tan and colleagues in 2004. These authors noted that loss of parafibromin nuclear staining had a high sensitivity and specificity for the definitive diagnosis of parathyroid carcinoma and their results were confirmed by other groups [41, 42].

Despite its specificity, other studies indicated that, although occurring more frequently in parathyroid carcinomas, loss of parafibromin nuclear staining can also occur in a small proportion of sporadic benign adenomas and therefore cannot be considered exclusive of malignancy. Curiously, these adenomas exhibit cystic features which are commonly observed in the HPT-JT syndrome [43]. Another important aspect is the fact that nuclear positivity cannot exclude the presence of *HRPT2* mutation, once some tumors harboring missense mutations revealed weak nuclear positivity [44].

Given the low frequency of parathyroid carcinomas, these are commonly misdiagnosed in the clinical setting and the need of specific markers for this disease is fundamental to reduce potential false-negative cases [45]. Some studies assessed the role of additional molecular marker to complement parafibromin staining in the screening process, emerging the protein 9.5 (PGP9.5) encoded by the *ubiquitin carboxyl-terminal esterase L1* (*UCHL1*), that was found to be upregulated in the majority of parathyroid carcinomas [46]. In contrast, the tumor suppressor *adenomatous polyposis coli* (*APC*) was found downregulated in carcinomas while benign lesions retain the expression [47].

In spite of these findings, the unequivocal diagnosis remains a challenge, and additional markers are likely to increase the knowledge and proper recognition of parathyroid carcinomas.

2. The Role of Other Genes in PHPT

The knowledge on the molecular bases of parathyroid tumorigenesis, particularly the sporadic variant, remains largely unknown. Some candidate genes have been studied because of their possible role in sporadic primary hyperparathyroidism development (see Table 1). Despite their involvement in familial forms, no somatic mutations have been found in *RET* and *CaSR* genes [48, 49].

Vitamin D receptor (VDR) has an important role in negatively controlling PTH secretion and parathyroid proliferation, thus representing a good target for this parathyroid pathological condition, but no mutations in either *VDR* gene or vitamin-D-activating enzyme were found in sporadic parathyroid tumors despite the evidence of the reduced expression in some series [62–65].

Abnormal Wnt signaling has been associated with many types of tumors, and deregulation of *CTNNB1* as well as mutations of *LRP5* coreceptor has been found in some series of parathyroid tumors. At variance, other studies pointed out that Wnt/beta-catenin signaling does not seem to contribute to the development of parathyroid tumors [66–68].

The existence of common molecular alterations among endocrine tumors and its proximity with *MEN1* gene

raised the hypothesis that *SDH5* gene could be involved in parathyroid tumorigenesis, but no genetic alterations were found in this pathology [69].

Using techniques such as comparative genomic hybridization (CGH), various authors verified the presence of chromosome gains and losses in specific regions, suggesting the presence of unidentified oncogenes and tumor suppressor genes, which may have a role in parathyroid tumorigenesis. Chromosomal regions in 1p, 6q, 9p, and 13q were found to be lost in benign and malignant parathyroid lesions, indicating these chromosomal regions as eventual carriers of novel tumor suppressor genes. Unknown oncogenes may be present in chromosomal regions of 9q, 16p, and 19p, because several authors demonstrated gain in these loci, in parathyroid tumors [70–72].

Chromosomal imbalances have been recognized as a mechanism able to alter the expression of miRNAs. Corbetta and collaborators explored the miR expression profile in parathyroid carcinomas, since the expression of these small noncoding RNAs varies between cancer and normal cells. These authors went to verify differential expression among parathyroid carcinoma and normal tissue. By real-time PCR, it was observed that the overall miRNA expression could separate normal versus cancer tissue and four miRs (miR-296, miR-139, miR-222, and miR-503) revealed differences between tumoural and normal parathyroid tissues [73]. Some genes such as *human growth factor-regulated tyrosine kinase substrate* (*HGS*) and *p27/Kip1* are described as potential targets of these miRNAs but further information about the biological relevance of these findings is needed and might provide tools for identifying new diagnostic and therapeutic strategies [74, 75].

Most of the studies in parathyroid tumorigenesis field have been performed using genomics and immunohistochemical approaches [53, 76]. Giusti and collaborators carried out comparative analysis to examine the changes in protein profile between adenomas and normal parathyroid tissue [77]. These authors verified the presence of 30 deregulated proteins in parathyroid adenomas, 22 of them being overexpressed when compared to normal parathyroid tissue [77]. Some of the identified proteins belong to the same functional class of the ones identified by Haven and co-workers when they preformed tissue microarrays for benign and malignant tumors [78]. Overall, these findings represent promising steps for the improvement of the knowledge about this pathology.

3. Concluding Remarks

Presently, alterations in *MEN1* and *CCND1* are still the main genetic alterations associated with the development of sporadic benign tumors (accounting for approximately 30% of the cases). The *HRPT2* gene is not only responsible for the HPT-JT syndrome but also mutated in the majority of parathyroid carcinomas.

A number of other candidate genes (due to their genomic localization, role in familial syndromes, and/or biological function) have been studied in parathyroid tumors, but without promising results.

Future goals include the identification of additional oncogenes and/or tumor suppressor genes in parathyroid lesions and understanding the molecular insights in the relationship between parathyroid proliferation and hormone regulation.

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Review Article

Cytogenetics and Molecular Genetics of Myxoid Soft-Tissue Sarcomas

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Myxoid soft-tissue sarcomas represent a heterogeneous group of mesenchymal tumors characterized by a predominantly myxoid matrix, including myxoid liposarcoma (MLS), low-grade fibromyxoid sarcoma (LGFMS), extraskeletal myxoid chondrosarcoma (EMC), myxofibrosarcoma, myxoinflammatory fibroblastic sarcoma (MIFS), and myxoid dermatofibrosarcoma protuberans (DFSP). Cytogenetic and molecular genetic analyses have shown that many of these sarcomas are characterized by recurrent chromosomal translocations resulting in highly specific fusion genes (e.g., *FUS-DDIT3* in MLS, *FUS-CREB3L2* in LGFMS, *EWSR1-NR4A3* in EMC, and *COL1A1-PDGFB* in myxoid DFSP). Moreover, recent molecular analysis has demonstrated a translocation $t(1; 10)(p22; q24)$ resulting in transcriptional upregulation of *FGF8* and *NPM3* in MIFS. Most recently, the presence of *TGFBR3* and *MGEA5* rearrangements has been identified in a subset of MIFS. These genetic alterations can be utilized as an adjunct in diagnostically challenging cases. In contrast, most myxofibrosarcomas have complex karyotypes lacking specific genetic alterations. This paper focuses on the cytogenetic and molecular genetic findings of myxoid soft-tissue sarcomas as well as their clinicopathological characteristics.

1. Introduction

Myxoid soft-tissue sarcomas encompass a heterogeneous group of rare tumors characterized by a marked abundance of mucoid/myxoid extracellular matrix. The main clinicopathological entities in this group are myxoid liposarcoma, low-grade fibromyxoid sarcoma, extraskeletal myxoid chondrosarcoma, myxofibrosarcoma, myxoinflammatory fibroblastic sarcoma, and myxoid dermatofibrosarcoma protuberans [1–4]. The correct classification of these sarcomas is important because of their distinct biological behaviors and potentially different treatments. However, it is often difficult to set apart many of these sarcomas due to overlapping histological features and lack of a distinct immunohistochemical profile. Moreover, the use of core needle biopsies to diagnose these sarcomas has become increasingly common, and this shift has created additional challenges.

Cytogenetic and molecular genetic assays are routinely used for diagnostic and prognostic purposes in molecular pathology laboratories [5]. Many of myxoid soft-tissue sarcomas are characterized by recurrent chromosomal translocations resulting in highly specific fusion genes [6, 7]. Advances in knowledge of the genetics of these sarcomas are leading to more accurate diagnosis. This paper reviews the cytogenetic and molecular genetic findings in these sarcoma types and their relationship with clinicopathological features. The consistent genetic alterations are summarized in Table 1.

2. Approaches to the Genetics of Soft-Tissue Sarcomas

Conventional karyotyping is the most comprehensive method for spotting the various translocations and other structural or numerical aberrations. It is dependent on the availability of fresh, sterile tumor tissue, the success of tumor cell growth in culture, and quality of metaphase cell preparations. When dividing cells are not available for cytogenetic studies, molecular approaches such as fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), reverse transcriptase-polymerase chain reaction (RT-PCR), or gene expression microarray can be used to evaluate genetic alterations.

TABLE 1: Chromosomal alterations and related molecular events in myxoid soft-tissue sarcomas.

Tumor type	Chromosomal alteration	Molecular event	Prevalence
Myxoid/round cell liposarcoma	$t(12; 16)(q13; p11)$	*FUS-DDIT3*	>90%
	$t(12; 22)(q13; q12)$	*EWSR1-DDIT3*	<5%
Low-grade fibromyxoid sarcoma	$t(7; 16)(q32–34; p11)$	*FUS-CREB3L2*	>95%
	$t(11; 16)(p11; p11)$	*FUS-CREB3L1*	<5%
Extraskeletal myxoid chondrosarcoma	$t(9; 22)(q22; q12)$	*EWSR1-NR4A3*	75%
	$t(9; 17)(q22; q11)$	*TAF15-NR4A3*	15%
	$t(9; 15)(q22; q21)$	*TCF12-NR4A3*	<1%
	$t(3; 9)(q12; q22)$	*TFG-NR4A3*	<1%
Myxofibrosarcoma	Complex karyotype	Not known	Not applicable
Myxoinflammatory fibroblastic sarcoma	$t(1; 10)(p22; q24)$	Deregulation of *FGF8* and *NPM3*	Not applicable
		Rearrangement of *TGFBR3* and *MGEA5*	Not applicable
Myxoid dermatofibrosarcoma protuberans	$t(17; 22)(q22; q13)*$	*COL1A1-PDGFB*	>90%

* Rearrangement also frequently seen as a ring chromosome.

FISH is the most helpful method for identifying specific gene rearrangements. It is more adaptable to formalin-fixed, paraffin-embedded tissues although imprint slides are preferred. Interphase FISH is particularly useful to assess intratumoral genetic heterogeneity as long as adequate combination of probes are used. FISH probes are readily available for a variety of relevant gene targets, including *DDIT3* (12q13), *FUS* (16p11), *EWSR1* (22q12), *FKHR* (13q14), *SYT* (18q11.2), and *ALK* (2p23) (Abbott Molecular Inc., Des Plaines, Ill, USA). It has been realized that FISH is a valuable adjunct in the diagnosis of myxoid soft-tissue tumors [8].

CGH is a method for genome-wide analysis of DNA sequence copy number in a single experiment. It maps the origins of amplified and deleted DNA sequences on normal chromosomes, thereby highlighting regions harboring potential oncogenes and tumor suppressor genes. However, CGH cannot detect rearrangements such as balanced translocations or inversions. Recently, a higher resolution version of CGH, so-called array CGH, has been made available. A distinct advantage of array CGH is the ability to directly map the copy number changes to the genome sequence. Similar to array CGH, single nucleotide polymorphism (SNP) array is capable of identifying small regions of chromosomal gains and losses at a high resolution. Also, SNP array can provide information regarding loss of hetero-zygosity.

RT-PCR is the most sensitive method to detect small numbers of translocation-bearing cells that are mixed within a tissue consisting of largely nonneoplastic cells. Sensitivity levels of 1 in a 100,000 cells are typically achieved. It may be suitable for the detection or monitoring of minimal residual disease [9]. However, the diagnostic success rate is variable and dependent on multiple factors. First, RNA quality may be inadequate because of RNA degradation. The second impediment of this methodology is the high risk of reagent contamination, mainly with PCR products, particularly in small laboratory spaces.

Microarray is a method for genome-wide monitoring of gene expression in a single experiment. A variety of commercial and noncommercial platforms can be used to perform global gene expression profiling. It is hoped that application of this technology will afford increased understanding of sarcoma biology and facilitate the development of new diagnostic markers and therapeutic agents [10–12].

Approximately one-third of all soft issue sarcomas exhibit a nonrandom chromosomal translocation. In addition, a subset of soft-tissue tumors carries specific oncogenic mutations (e.g., *KIT* or *PDGFRA* mutations in gastrointestinal stromal tumor). FISH and RT-PCR are commonly applied for the detection of specific genetic alterations in the differential diagnosis of soft-tissue sarcomas.

3. Myxoid Liposarcoma

The working group of the World Health Organization (WHO) for classification of tumors of soft-tissue and bone combined myxoid and round cell liposarcomas under myxoid liposarcoma (MLS) [13]. MLS is the second most common subtype of liposarcoma, representing approximately one-third of all liposarcomas. MLS occurs predominantly in the deep soft-tissues of lower extremities and has a peak incidence in the fourth and fifth decades of life with no gender predilection. Pure MLS is considered low-grade and has a 5-year survival rate of 90% [14]. In contrast, MLS containing a greater than 5% round cell component is considered high-grade and has a worse prognosis. The clinical outcome of multifocal MLS is poor [15]. In contrast to other soft-tissue sarcomas, MLS tends to metastasize to unusual sites such as retroperitoneum, opposite extremity, and bone.

Histologically, pure MLS is composed of primitive mesenchymal cells in a myxoid matrix, often featuring mucinous pools (Figure 1(a)). Lipoblasts are most often univacuolated, small, and tend to cluster around vessels or at the periphery of the lesion. A delicate plexiform capillary vascular network is present and provides an important clue for distinguishing MLS from intramuscular myxoma [16]. A subset of MLS shows histological progression to hypercellular or round cell morphology. The round cell areas are characterized by solid sheets of primitive round cells with a high nuclear/cytoplasmic ratio and a prominent nucleolus. Pure round cell liposarcoma is extremely rare and may be confused with
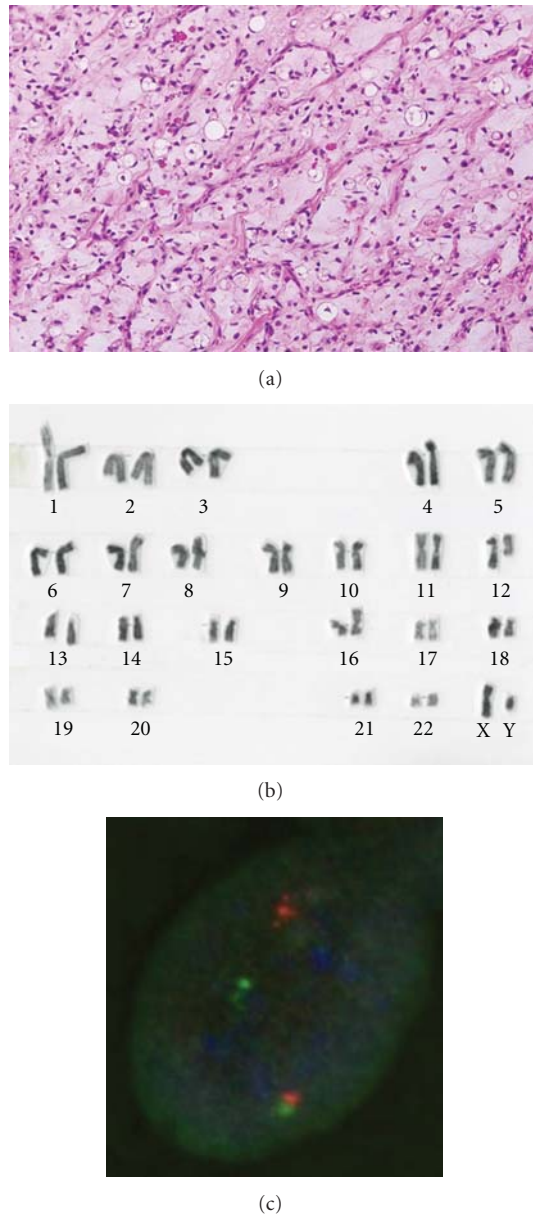
(a)

(b)

(c)

FIGURE 1: (a) Myxoid liposarcoma with a myxoid background containing a delicate arborizing capillary vascular network, small uniform mesenchymal cells, and lipoblasts. (b) G-banded karyotype showing a 12;16 translocation as the sole aberration. (c) Fluorescence in situ hybridization analysis using a *DDIT3* (12q13) break-apart probe shows a split of the orange and green signals, indicating a disruption of the *DDIT3* locus.

other round cell sarcomas such as Ewing sarcoma/primitive neuroectodermal tumor, rhabdomyosarcoma, and poorly differentiated synovial sarcoma.

MLS is characterized by a recurrent translocation $t(12;16)(q13;p11)$ in more than 90% of cases (Figure 1(b)), which fuses the 5′ portion of the *FUS* gene on chromosome 16 with entire reading frame of the *DDIT3* gene on chromosome 12 [17–19]. A small percentage of cases carry a variant translocation $t(12;22)(q13;q12)$ resulting in an *EWSR1-DDIT3* fusion gene [15, 20–28]. The presence of these translocations

and molecular alterations is highly sensitive and specific for MLS. Therefore, cytogenetics is an excellent analytic method for the initial workup of a suspected MLS. FISH and RT-PCR can also be used to provide support for the diagnosis of MLS (Figure 1(c)) [8, 29–32]. In addition, several nonrandom secondary alterations have been identified, including 6q deletion, isochromosome 7q10, trisomy 8, and unbalanced 1;16 translocation [17, 24, 33–35]. Conventional and array CGH studies have shown gains of 8p21–23, 8q, and 13q [36–38].

To date, 12 *FUS-DDIT3* and four *EWSR1-DDIT3* variants of fusion transcripts have been described in MLS [22, 26, 28, 39, 40]. Most cases of MLS are one of three different *FUS-DDIT3* fusion transcript types, including varying portions of *FUS*. The *FUS-DDIT3* fusion transcript type does not appear to have a significant impact on clinical outcome [22, 26]. On the other hand, Suzuki et al. [28] reported that MLS with a type 1 *EWSR1-DDIT3* fusion transcript may show more favorable clinical behavior than MLS with other types of fusion transcripts. Interestingly, clinical data suggest that the fusion transcript type may influence response to therapy with trabectedin [41].

Several receptor tyrosine kinases (RTKs) are highly expressed in MLS, including RET, MET, and IGF1R [42, 43]. These RTKs promote cell survival and cell proliferation through the PI3K/AKT and the Ras-Raf-ERK/MAPK pathways [42]. Recently, Barretina et al. [44] demonstrated that mutation of *PIK3CA*, encoding the catalytic subunit of PI3K, is associated with AKT activation and poor clinical outcome. AKT activation functions as a master switch to generate a plethora of intracellular signals and intracellular responses and is more frequent in the round cell variant [43]. It has also been shown that the NF-κB pathway is highly active in MLS [40]. Moreover, Göransson et al. [45] showed that NF-κB is a major factor controlling *IL8* transcription in FUS-DDIT3-expressing cells. NF-κB is an inducible cellular transcription factor that regulates a variety of cellular genes, including those involved in immune regulation, inflammation, cell survival, and cell proliferation. These findings will help to develop new potential therapeutic strategies for MLS patients with advanced disease.

4. Low-Grade Fibromyxoid Sarcoma

Low-grade fibromyxoid sarcoma (LGFMS), first described by Evans [46] in 1987, is a rare but distinctive fibromyxoid variant of fibrosarcoma. It includes the tumor originally designed as hyalinizing spindle cell tumor with giant rosettes [47]. LGFMS occurs primarily in young to middle-aged adults with a male predominance, but this tumor may affect children [48, 49]. LGFMS typically presents as a slowly growing, painless mass in the deep soft-tissues of lower extremities or trunk. Local recurrence and metastatic rates are 9%–21% and 6%–27%, respectively [49, 50]. The overall prognosis for superficial LGFMS appears to be better than that for deep LGFMS [48].

Histologically, LGFMS shows alternating fibrous and myxoid areas with bland spindle-shaped cells arranged in a whorled pattern (Figure 2). Cellularity is variable but generally low and mitoses are scarce. There is often a prominent

network of branching capillary-sized blood vessels reminiscent of myxoid liposarcoma. Approximately 40% of cases have giant collagen rosettes characterized by a central zone of eosinophilic collagen surrounded by a palisade of round to oval tumor cells [13]. This variant was originally termed hyalinizing spindle cell tumor with giant rosettes [47]. Immunohistochemically, the tumor cells are diffusely positive for vimentin and focally for epithelial membrane antigen (EMA) [48, 50]. Immunostains for S-100 protein, desmin, and CD34 are typically negative.

LGFMS is characterized by a recurrent balanced translocation $t(7; 16)(q34; p11)$ resulting in an *FUS-CREB3L2* fusion gene [50–53]. This same translocation was identified in cases of hyalinizing spindle cell tumor with giant rosettes [54, 55], suggesting a pathogenetic link between these two entities. A small percentage of cases carry a variant translocation $t(11; 16)(p11; p11)$ leading to a fusion of the *FUS* and *CREB3L1* genes [50, 53]. Interestingly, supernumerary ring chromosomes have been observed as the sole anomaly in a subset of LGFMS [52, 56, 57]. FISH and CGH studies have demonstrated that ring chromosomes are composed of material from chromosomes 7 and 16 [56, 57]. Bartuma et al. [57] showed that the *FUS-CREB3L2* fusion gene can be present in ring chromosomes.

The breakpoints in the fusion transcripts are mostly at exon 6 or 7 of *FUS* and exon 5 of *CREB3L2* or *CREB3L1* [50–53, 58]. CREB3L2 is a member of CREB3 family of transcription factors and contains a basic DNA-binding and leucine zipper dimerization domain, highly similar to that in CREB3L1. Panagopoulos et al. [59] suggested that the FUS-CREB3L2 fusion protein is a more potent transcriptional activator than the native CREB3L2 and may contribute to the pathogenesis of LGFMS through the deregulation of its target genes. The molecular variability of fusion transcripts in LGFMS does not appear to have a significant impact on microscopic appearances or clinical outcome [53].

5. Extraskeletal Myxoid Chondrosarcoma

Extraskeletal myxoid chondrosarcoma (EMC) is categorized by the WHO as a tumor of uncertain differentiation, because there is a paucity of convincing evidence of cartilaginous differentiation [13]. Most EMCs arise in the deep soft-tissues of the proximal extremities and limb girdles, especially the thigh and popliteal fossa, similar to MLS. EMC has a peak incidence in the fifth and six decades of life with a male predominance. Only a few cases have been encountered in children and adolescents [60–62]. Patients typically present with a slowly growing mass that causes pain or tenderness in approximately one-third of cases [16]. Local recurrence and metastatic rates are 48% and 46%, respectively [61]. EMC has a 10-year survival rate of 63%–88%, but a 10-year disease-free survival is much lower, ranging from 14% to 36% [61, 63–66]. Large tumor size (especially >10 cm), advanced age, and proximal tumor location appear to be poor prognostic factors in EMC [61, 63, 67].

Histologically, EMC is characterized by multinodular growth of a cord-like or lace-like arrangement of round or slightly elongated cells in an abundant myxoid matrix (Fig-
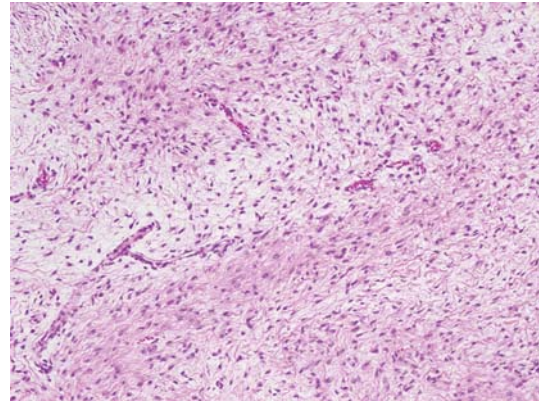

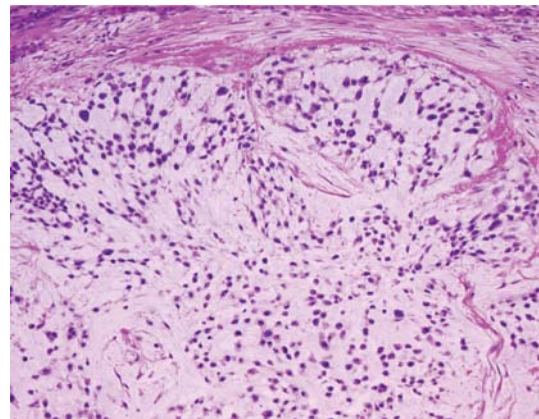FIGURE 2: Low-grade fibromyxoid sarcoma with alternating fibrous and myxoid areas.

FIGURE 3: Extraskeletal myxoid chondrosarcoma with an abundant myxoid matrix containing round or slightly elongated cells with small hyperchromatic nuclei.

ure 3). The tumor cells have small hyperchromatic nuclei and a narrow rim of deeply eosinophilic cytoplasm. Occasional cells show cytoplasmic vacuolization [16]. Mitotic figures are rare in most cases. In contrast to the bland-looking or low-grade morphology, cellular or high-grade EMC has also been described [61, 68, 69]. Some authors have suggested that the cellular or high-grade EMC is likely to have a worse prognosis than conventional EMC [63, 68, 70] although its prognostic significance has not yet been established [67]. Immunohistochemically, vimentin is the only marker consistently positive in EMC. S-100 protein is expressed in approximately 30% of cases [67], often with focal and weak immunoreactivity. Only a small percentage of cases may show scattered cells that are EMA positive [67]. Recent immunohistochemical and ultrastructural studies have demonstrated that some EMCs may have neuroendocrine differentiation [63, 69, 71].

EMC is characterized by a recurrent translocation $t(9; 22)(q22; q12)$ in approximately 75% of cases, which fuses the *EWSR1* gene on 22q12 with the *NR4A3* gene on 9q22 [72–78]. A second variant translocation, $t(9; 17)(q22; q11)$, has been detected in approximately 15% of EMC and results in a *TAF15-NR4A3* fusion gene [78–82]. In addition, two

additional variant translocations, $t(9; 15)(q22; q21)$ resulting in a *TCF12-NR4A3* fusion gene and $t(3; 9)(q12; q22)$ resulting in a *TFG-NR4A3* fusion gene, have also been identified, each only in a single case [83, 84]. Because these fusion genes have not yet been described in any other tumor type, they represent useful diagnostic markers. Moreover, several nonrandom secondary alterations have been identified in approximately 50% of cytogenetically analyzed cases, including gain of 1q and trisomy for chromosomes 7, 8, 12, and 19 [77, 78]. The biological significance of these chromosomal alterations remains unknown.

Two main *EWSR1-NR4A3* fusion transcript types have been reported for the $t(9; 22)(q22; q12)$ in EMC [69, 77, 78]. The most common fusion transcript contains exon 12 of *EWSR1* fused to exon 3 of *NR4A3* (type 1), whereas exon 7 of *EWSR1* is fused to exon 2 of *NR4A3* in the type 2 fusion transcript. In the *TAF15-NR4A3* fusion transcript, exon 6 of *TAF15* is fused exclusively to exon 3 of *NR4A3* [77]. NR4A3 is a member of NR4A subfamily within the nuclear receptor superfamily and contains a zinc finger DNA-binding domain. The EWSR1-NR4A3 fusion protein is thought to function as a potent transcriptional activator for *NR4A3*-target genes [85, 86]. It has also been shown that the TAF15-NR4A3 fusion protein functions a strong transcriptional activator [87]. It is unclear whether the fusion transcript type is associated with particular morphological features or clinical outcome.

Gene expression profiling studies of EMC have revealed overexpression of the *CHI3L1*, *METTL1*, *RELB*, *MYB*, *NMB*, *DKK1*, *DNER*, *CLCN3*, *DEF6*, *NDRG2*, and *PPARG* genes [78, 88, 89]. In addition, several genes encoding neural-neuroendocrine markers have been expressed, including *SCG2*, *NEF3*, *GFAP*, *GAD2*, *ENO2*, *SYP*, *CHGA*, *NEF3*, and *INSM1* [78, 88]. *CHI3L1* encodes a glycoprotein member of the glycosyl hydrolase 18 family, which is secreted by activated chondrocytes, macrophages, neutrophils, and synovial cells. Sjögren et al. [78] suggested that CHI3L1 may be useful as a serum marker monitoring disease progression in EMC patients. NMB is a member of bombesin-related peptide family in mammals and a secreted protein involved in stimulation of smooth muscle contraction [90]. Subramanian et al. [88] suggested that NMB may prove to be a serological marker of EMC recurrence. *DKK1* encodes a protein that is a member of the dickkopf family. DKK1 is involved in embryonic development through its inhibition of the WNT signaling pathway. Because DKK1 is a secreted protein, it may serve as a prognostic marker for evaluation of EMC. *PPARG* encodes a member of the peroxisome proliferator-activated receptor subfamily of nuclear receptors. PPARG is known as a regulator of adipocytic differentiation [91]. Interestingly, Filion et al. [89] demonstrated that *PPARG* is the first direct transcriptional target of the EWSR1-NR4A3 fusion protein. These findings will lead to the development of molecularly targeted therapies for patients with advanced EMC.

6. Myxofibrosarcoma

Myxofibrosarcoma, formerly known as myxoid malignant fibrous histiocytoma (MFH), is now defined as a distinct histological entity [13]. It is one of the most common soft-tissue sarcomas in elderly patients. Most myxofibrosarcomas arise in the dermal and subcutaneous tissues of the limbs (especially lower limbs) and limb girdles. Myxofibrosarcoma has a peak incidence in the sixth to eighth decades of life with a slight male predominance. Patients typically present with a slowly growing, painless mass. Recently, an epithelioid variant of myxofibrosarcoma with an aggressive course has been described [92].

Grading of myxofibrosarcoma is somewhat controversial. Myxofibrosarcoma has been subdivided into three or four grades based on the degree of cellularity, nuclear pleomorphism, and mitotic activity [93, 94]. Local recurrences occur in up to 50% to 60% of cases [93–95], irrespective of histological grade. Whereas low-grade myxofibrosarcomas usually do not metastasize, intermediate and high-grade lesions may develop metastases in approximately 16% to 38% of cases [93–95]. Importantly, low-grade myxofibrosarcomas may become higher grade in subsequent recurrences and acquire metastatic potential. The overall 5-year survival rate is 60%–70% [13].

Histologically, myxofibrosarcoma is characterized by multinodular growth of spindle or stellate-shaped cells within variably myxoid stroma containing elongated, curvilinear blood vessels (Figure 4). The tumor cells have slightly eosinophilic cytoplasm and mildly atypical, hyperchromatic nuclei. Vacuolated cells with cytoplasmic acid mucin, mimicking lipoblasts, are also seen [13]. Mitotic figures are rare in low-grade lesions. In contrast, high-grade myxofibrosarcomas are composed of solid sheets and fascicles of atypical spindled and pleomorphic tumor cells with hemorrhagic and necrotic areas. Bizarre, multinucleated giant cells are also occasionally found. Mitotic figures, including abnormal mitoses, are frequent. At least focally, however, areas of a lower grade neoplasm with a prominent myxoid matrix are present [13]. Intermediate-grade myxofibrosarcomas are more cellular than low-grade lesions and often contain minute solid areas showing flank pleomorphism. Immunohistochemically, the tumor cells are diffusely positive for vimentin and occasionally for muscle specific actin and α-smooth muscle actin, suggestive of focal myofibroblastic differentiation.

Data on the cytogenetics and molecular genetics of myxofibrosarcoma are difficult to evaluate, because the diagnostic criteria for this tumor have changed with time. In general, myxofibrosarcomas are associated with highly complex karyotypes lacking specific structural aberrations [96–98]. The only recurrent gain involves chromosome 7, whereas losses primarily affect chromosomes 1, 3, 5, 6, 10, 12, 16, 17, and 19 [7]. The presence of ring chromosomes has been described in some cases of low-grade myxofibrosarcoma (or myxoid MFH) [98–100]. In addition, homogeneously staining regions, double minutes, and marker chromosomes have been found. Recently, Willems et al. [98] proposed the concept of progression of myxofibrosarcoma as a multistep genetic process ruled by genetic instability.

A conventional CGH study of 22 myxofibrosarcomas showed gains of 19p and 19q, losses of 1q, 2q, 3p, 4q, 10q, 11q, and 13q, and high-level amplifications of the central

regions of chromosome 1, 5p, and 20q [101]. Interestingly, gain of 5p and loss of 4q are not observed in low-grade myxofibrosarcomas as opposed to higher grade neoplasms, suggesting that these aberrations are late events in the oncogenesis of myxofibrosarcoma. In addition, array CGH studies showed gains of 7p21-22, 7q31–35, and 12q15–21 and losses of 10p13-14, 10q25-26, and 13q14–34 [38, 102, 103]. These findings suggest that loss of chromosome 13q is the most frequent genomic imbalance in myxofibrosarcoma, leading to inactivation of the RB pathway.

Recently, Lee et al. [103] reported that MET is expressed in approximately two-third of cases and its overexpression is highly related to deep location, higher grades, and more advanced stages. The authors suggested that MET may represent a target of choice to develop novel therapeutic strategies for myxofibrosarcoma.

A recent gene expression analysis has shown that the *WISP2*, *GPR64*, and *TNXB* genes are upregulated in myxofibrosarcoma compared with other spindle cell and pleomorphic sarcomas [104]. *WISP2* encodes a member of the WNT1 inducible signaling pathway protein subfamily, which belongs to the connective tissue growth factor family. WISP2 is a secreted protein involved in several important human diseases or conditions that are marked by aberrant cell proliferation and migration [105]. GPR64 is a highly conserved, tissue-specific, seven-transmembrane receptor of the human epididymis [106]. *TNXB* encodes a member of the tenascin family of extracellular matrix glycoproteins. TNXB is thought to function in matrix maturation during wound healing, and its deficiency is associated with the connective tissue disorder Ehlers-Danlos syndrome [107]. Nakayama et al. [104] suggested that these genes may serve as novel diagnostic markers for myxofibrosarcoma. Most recently, Barretina et al. [44] demonstrated that *NF1* is mutated or deleted in 10.5% of myxofibrosarcomas.

7. Myxoinflammatory Fibroblastic Sarcoma

Myxoinflammatory fibroblastic sarcoma (MIFS), also known as inflammatory myxohyaline tumor of the distal extremities with virocyte or Reed-Sternberg-like cells, is a recently described soft-tissue tumor entity [108, 109]. MIFS occurs predominantly in the subcutaneous tissues of distal extremities and has a peak incidence in the fourth and fifth decades of life with no gender predilection. Patients typically present with a slowly growing, painless, ill-defined mass. The preoperative diagnosis in most cases is benign and may include tenosynovitis, ganglion cyst, and giant cell tumor of tendon sheath [13]. Local recurrence and metastatic rates are 31.3% and 3.1%, respectively [110].

Histologically, MIFS is multinodular, poorly delineated, and characterized by a prominent myxoid matrix containing numerous inflammatory cells, including lymphocytes, plasma cells, neutrophils, and eosinophils [109]. Germinal centers are occasionally encountered. Neoplastic cells include spindle-shaped and epithelioid cells with mild to moderate nuclear atypia, large polygonal and bizarre ganglion-like cells, Reed-Sternberg-like cells with huge inclusion-like
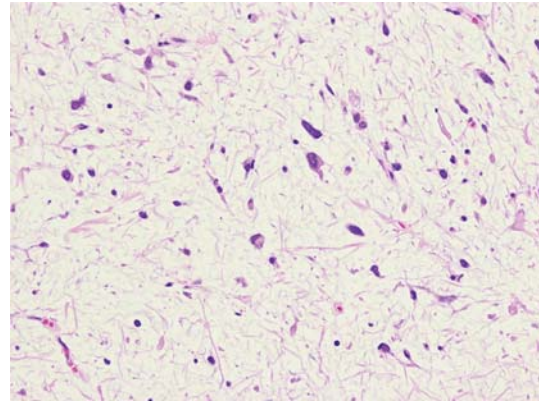

FIGURE 4: Myxofibrosarcoma with a myxoid stroma containing spindle or stellate-shaped cells with mildly atypical, hyperchromatic nuclei.
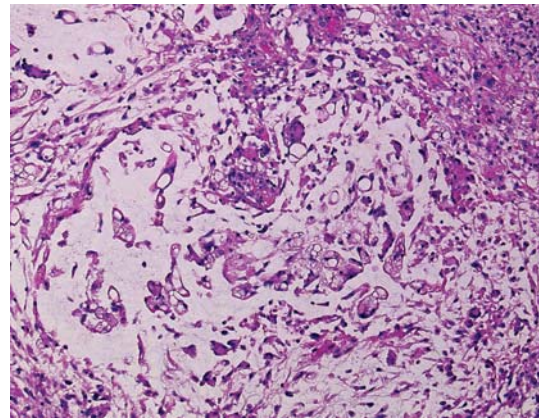

FIGURE 5: Myxoinflammatory fibroblastic sarcoma with a myxoid background containing spindle-shaped and epithelioid cells, inflammatory cells, and pseudolipoblasts.

nucleoli, and multivacuolated lipoblast-like cells (Figure 5). Hemosiderin deposition may be conspicuous. Mitotic activity is usually low, and necrosis is rarely present. Immunohistochemically, the tumor cells are diffusely positive for vimentin and focally for CD68 and CD34 [16]. Occasional cases may show scattered cells that stain for cytokeratin or α-smooth muscle actin. Immunostains for S-100 protein, HMB-45, desmin, EMA, leukocyte common antigen, CD15, and CD30 are typically negative.

Cytogenetic and molecular cytogenetic studies have identified the frequent presence of a balanced or unbalanced $t(1; 10)(p22; q24)$ translocation and ring chromosomes containing amplified material from the 3p11-12 region in MIFS [111–113]. A balanced translocation, $t(2; 6)(q31; p21.3)$, has also been described as the sole anomaly in a single case [114]. Most recently, Antonescu et al. [115] demonstrated the presence of *TGFBR3* (1p22) and *MGEA5* (10q24) gene rearrangements by FISH in a subset of MIFS. It is of interest that the $t(1; 10)$ translocation and these gene rearrangements have also been identified in hemosiderotic fibrolipomatous tumor (HFLT) [113, 115–117]. These findings suggest that

MIFS and HFLT may represent different morphologic variants of the same entity.

Conventional and array CGH studies showed amplification of 3p11-12 [113, 118]. Notably, Hallor et al. [113] demonstrated that 3p11-12 amplification is associated with an increased expression of *VGLL3* and *CHMP2B*. *VGLL3* encodes a protein that is a cofactor of transcription factors of the TEAD family. It has also been shown that *VGLL3* is amplified and overexpressed in myxofibrosarcoma, undifferentiated pleomorphic sarcoma, and dedifferentiated liposarcoma [119]. These findings strongly suggest that *VGLL3* is the main target of 3p11-12 amplification and this genetic event plays an important role in the development and progression of certain subsets of soft-tissue sarcomas.

A recent gene expression analysis has shown that the *FGF8* and *NPM3* genes are upregulated in the $t(1;10)$ -positive tumors compared with tumors without such a translocation [113]. These two genes downstream of *MGEA5* have been mapped to 10q24. FGF8, a member of the fibroblast growth factor family, is a secreted heparin-binding protein, which has transforming potential. FGF8 is widely expressed during embryonic development. Overexpression of *FGF8* has been shown to increase tumor growth and angiogenesis [120]. Hallor et al. [113] suggested that deregulation of *FGF8* may constitute an important event in the development of a subset of MIFS.

8. Myxoid Dermatofibrosarcoma Protuberans

Myxoid dermatofibrosarcoma protuberans (DFSP) is a rare but distinctive variant of DFSP with a prominent myxoid matrix. Clinically, myxoid DFSP is similar to typical DFSP [121–123]. DFSP occurs primarily young to middle-aged adults with a male predominance, but this tumor may affect children, including congenital occurrence [124]. It typically presents as a slowly growing, plaque-like or small nodular lesion. The most common location is the trunk, followed by the limbs and head and neck. Local recurrence and metastatic rates are 0%–52% and 0%–1.7%, respectively [125]. The overall prognosis of typical DFSP is excellent if completely excised with negative microscopic margins. Reimann and Fletcher [122] stated that myxoid DFSP appears to have a similarly good prognosis. Recognition of this DFSP variant is important to avoid misdiagnosis of more or less aggressive myxoid soft-tissue tumors.

Histologically, myxoid DFSP is characterized by a sheet-like to vaguely lobular proliferation of bland spindle cells in an abundant myxoid stroma (Figure 6). The tumor cells have slightly eosinophilic cytoplasm and stellate to oval nuclei with indistinct nucleoli. Branching, thin-walled blood vessels are frequently present. All cases display at least focally a strikingly infiltrative growth pattern, with trapping of subcutaneous adipose tissue in the characteristic honeycomb manner also observed in typical DFSP [122]. Mitotic activity is usually low. Immunohistochemically, the tumor cells are diffusely positive for vimentin and CD34. Immunostains for S-100 protein, desmin, muscle specific actin, α-smooth muscle actin, cytokeratin, and EMA are typically negative. Apolipoprotein D (APOD) has been found
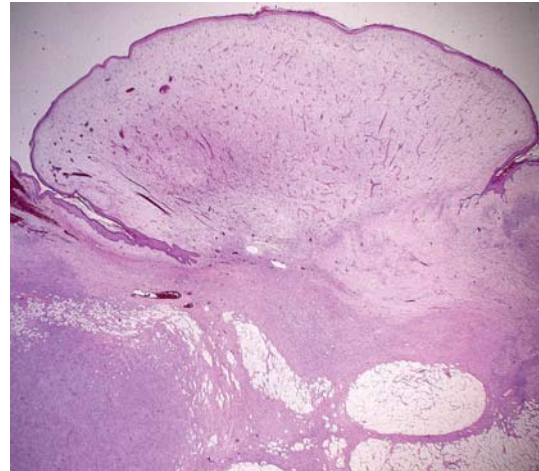

Figure 6: Typical example of a myxoid dermatofibrosarcoma protuberans.

to be highly expressed in DFSP and its histological variants [126].

DFSP is characterized by an unbalanced translocation $t(17;22)(q22;q13)$, which fuses the *COL1A1* gene on 17q21-22 with the *PDGFB* gene on 22q13 [127–130]. The same molecular event is also seen in supernumerary ring chromosomes derived from the $t(17;22)$ [129, 130]. Identical genetic changes have also been shown in the histological variants, including myxoid DFSP [123], pigmented DFSP (Bednar tumor) [131], Granular cell DFSP [132], juvenile variant of DFSP (giant cell fibroblastoma) [128], and fibrosarcomatous variant of DFSP [133, 134]. Other rare translocations, including $t(X;7)$, $t(2;7)$, $t(9;22)$, and $t(5;8)$, have also been described [135–138]. Moreover, several secondary nonrandom alterations have been identified, including trisomy 5 and trisomy 8 [130]. The clinical and biological implications of these chromosomal alterations are virtually unknown.

Conventional and array CGH studies showed gain or high-level amplification of 17q and 22q in most cases [139–141]. DFSP is occasionally misdiagnosed as benign lesions such as dermatofibroma, leading to improper primary management. We suggested that CGH may be a useful diagnostic tool for distinguishing DFSP from dermatofibroma [140]. The presence of gain in 8q was also observed [140–142]. Interestingly, FISH and CGH studies have indicated an association between an increased number of *COL1A1-PDGFB* genomic copies and fibrosarcomatous transformation in a subset of DFSP [139, 143, 144]. Most recently, Salgado et al. [145] reported that the majority of DFSP harbor the *COL1A1-PDGFB* fusion and FISH should be recommended as a routine diagnostic tool.

The breakpoint of *PDGFB* is remarkably constant (exon 2). In contrast, the *COL1A1* breakpoint may occur in any of the exons in the α-helical coding region (exons 6–49). The most frequently rearranged *COL1A1* exons are exon 25, 32, and 47 [146]. *PDGFB* encodes the β chain of platelet-derived growth factor. PDGFB is a potent mitogen for

a variety of cells [147]. *COL1A1* encodes the pro-α1 chains of type I collagen whose triple helix comprises two α1 chains and one α2 chain. Type I collagen is a major structural protein found in the extracellular matrix of connective tissue such as skin, bone, and tendon. The COL1A1-PDGFB fusion protein is posttranslationally processed to a functional PDGFB, and results in PDGFB-mediated autocrine and/or paracrine activation of PDGFRB [128, 148]. Inhibitors of PDGFRB, such as imatinib mesylate, have been reported to show clinical activity for metastatic or locally advanced DFSP [149–151]. These results support the concept that DFSP cells are dependent on aberrant activation of PDGFRB for cellular proliferation and survival. No correlation between the molecular subtype of *COL1A1-PDGFB* fusion gene and the clinicopathological features has been established [146, 152].

Gene expression profiling studies of DFSP have revealed overexpression of the *PDGFB*, *PDGFRB*, *APOD*, *SPRY2*, *NRP1*, *EGR2*, and *MEOX1* genes [10, 153]. *SPRY2* encodes a protein belonging to the sprouty family and is involved in the regulation of the EGF, FGF, and Ras/MAPK signaling pathways. NRP1 is a membrane-bound coreceptor to a tyrosine kinase receptor for both vascular endothelial growth factor and semaphorin family members and plays a role in angiogenesis, cell survival, migration, and invasion. EGR2 is a transcription factor with three tandem C2H2-type zinc fingers and plays a role in the PTEN-induced apoptotic pathway [154]. *MEOX1* has been mapped to 17q21 and encodes a member of a subfamily of nonclustered, diverged, antennapedia—like homeobox—containing genes. The homeobox genes are involved in early embryonic development and the determination of cell fate. Linn et al. [153] proposed the possibility that DFSP are derived from early embryonic mesenchymal cells.

9. Conclusions

It is important to be familiar with the clinicopathological and molecular genetic features of myxoid soft-tissue sarcomas for their accurate diagnosis and appropriate treatment. In our experience, FISH is a valuable ancillary diagnostic tool for these sarcomas, especially on limited tissue samples. Novel diagnostic and/or prognostic molecular markers as well as promising therapeutic targets have gradually been recognized. In the future, treatment decisions and prognosis assessment for myxoid soft-tissue sarcomas will increasingly be based on a combination of histological criteria and molecular identification of genetic alterations indicative of biological properties.

Acknowledgment

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