Disease Markers 23 (2007) 105–112
IOS Press

Loss of imprinting of *IGF2* as an epigenetic marker for the risk of human cancer

Hengmi Cui*
Division of Molecular Medicine, Department of Medicine, Johns Hopkins University School of Medicine, 1064 Ross/720 Rutland Avenue, Baltimore, MD 21205, USA

Abstract. *IGF2* is the first gene discovered to be imprinted and expressed exclusively from the paternal allele in both human and mouse. *IGF2* is also the first imprinted gene displaying loss of imprinting (LOI) or aberrant imprinting in human cancers. Evidently, LOI or reactivation of the maternal allele of *IGF2* is associated with an increase of *IGF2* expression that may subsequently play an important role in the onset of human cancers. The most important discovery was the association of LOI of *IGF2* with the risk of developing human colorectal cancer. LOI occurs not only in colon cancer tissues, but also in matched normal tissues and peripheral blood cells. A pilot study indicated a significant relationship between LOI of *IGF2* and family history as well as personal history of colorectal cancer, suggesting that LOI of *IGF2* might be a valuable biomolecular marker of predicting an individual's risk for colon cancer. A recent epigenetic progenitor model suggested that human cancers might have a common basis that involves an epigenetic disruption of progenitor cells mediated by "tumor progenitor genes" and proposed that non-neoplastic but epigenetically disrupted progenitor cells might be an important target for cancer risk assessment and prevention.

Keywords: *IGF2*, loss of imprinting, cancer, biomarker

1. Introduction

Genomic imprinting is an important phenomenon brought about through the allelic-specific epigenetic modification leading to the parent-of-origin specific gene expression. Genomic imprinting was initially suggested based on mouse oocyte manipulation. The idea that parthenogenetic animals with homozygous genomes would be useful in detecting harmful recessive genes in animal breeding applications impelled scientists to produce such animals in the laboratory [20]. Unfortunately, these attempts were unsuccessful. Nevertheless, it was found that unfertilized mouse eggs could be stimulated to begin division without the need of fertilization and that diploids could be formed by the suppression of division II of meiosis using the drug cytochallasin B. However, parthenogenone embryos were unable to develop fully after being injected into foster mothers although they could be aggregated with normal mouse eggs to form chimeras which were capable of developing into adulthood [53]. In 1984, results from two separate research groups provided evidence that revealed the paternal and maternal genomes as functional non-equivalents. Through experimentally manipulating one-cell embryos, the two research groups suggested that the presence of both paternal and maternal genome are essential for normal development and genomic imprinting in mammalian [30,55].

The IGF2 gene encodes insulin-like growth factor II, which is structurally homologous to insulin and displays growth promoting and metabolic effects on various cell types. *IGF2* imprinting in mouse was discovered from an experiment that targeted the disruption of the IGF2 gene in which transmission of the *IGF2* mutation through the male germline resulted in heterozygous progenies that were growth deficient, while transmission of the disrupted gene through the maternal germline resulted in heterozygous offspring that were phenotypically normal. Molecular biologi-

*Corresponding author. Tel.: +1 410 502 4081; Fax: +1 410 614 9819; E-mail: hcui2@jhmi.edu.

ISSN 0278-0240/07/$17.00

cal analysis further indicated that only the paternal allele is expressed in embryos, while the maternal allele is silent [12]. The discovery of human *IGF2* imprinting was derived from the studies of Wilms' tumor and Beckwith-Wiedemann syndrome (BWS) patients in three independent laboratories [39,41,46]. Using restriction fragment length polymorphism (RFLP) and RNAase protection analysis, researchers discriminated the parental alleles of transcripts of *IGF2* and found that *IGF2* is expressed exclusively from paternal alleles. Not only were these results precisely consistent with those observations found in mouse, more importantly, two of the three laboratories occasionally found the loss of imprinting of *IGF2*, or the re-activation of maternal allele of *IGF2* in human Wilms' tumors [39,46]. These results, published in the same issue of *Nature*, were the first to associate aberrant imprinting with human tumor. Clearly, the discoveries of genomic imprinting and loss of imprinting depend largely on the development of modern molecular biological techniques.

2. Laboratory methods for *IGF2* imprinting analysis

One of the most important characteristics of imprinted genes is its allelic-specific transcription. Imprinted genes could not be identified until modern molecular biology techniques enabled the discrimination of the two parental alleles of imprinted genes on the transcriptional level. Here I will describe several major methods for the examination of human *IGF2* imprinting.

2.1. Restriction Fragment Length Polymorphism (RFLP) method

Human *IGF2* has an *Apa I* digestion single nucleoside polymorphism (SNP) within the exon 9. Both the Feinberg and Reeve laboratories used the RFLP method to identify *IGF2* as an imprinted gene which is transcribed exclusively from the paternal allele while the maternal allele remains silent in normal human fetal tissues [39,46]. They first screened informative or heterozygous samples by running PCR on genomic DNA and then digested the PCR products with the *Apa I* enzyme. Next, RNA from the informative sample was reverse-transcribed and amplified using PCR. The PCR product was again digested with *Apa I*. Notably it is essential to treat RNA with DNase before reverse transcription to eliminate any contaminated genomic DNA for it can bias the analyzed imprinting result. Thus, in order to overcome any DNA contamination interference during imprinting analysis, Cui et al. treated RNA samples with DNAse before reverse transcription [7]. At the same time, PCR primers were designed across an intron-exon boundary (exon 8-exon 9). This strategy easily distinguished whether the PCR products were derived from RNA or genomic DNA since the size of PCR products from genomic DNA is longer than that from cDNA. For the *IGF2* imprinting quantitative analysis, one primer was labeled with isotope and hot PCR products were digested with *Apa I* and then soluted on 6% polyacrylamide and finally quantified on a Phosphorimager. The quantitative ratio of more abundant to less-abundant allele was then calculated. This method is simple and quick and has been widely applied by many laboratories. The only limitation is that it can only be used to check *Apa I* informative samples and the results obtained are only semi-quantitative. Later, Uejima et al further improved the RFLP method by the use of Hot-stop PCR, a simple and quantitative technique for the measurement of allele ratios that circumvents the problem of heteroduplex formation skewing the results of *Apa I* restriction endonuclease digestion of PCR products [58]. The strategy allowed a 5' end-labelled primer to be added to the PCR reaction right before the last PCR step. After hot primer extension and restriction endonuclease digestion, the reaction product could finally be visualized with the exclusion of any possibilities of heteroduplexes of PCR products biasing imprinting results.

2.2. Ribonuclease Protection Assays (RPA) method

RPA is a sensitive technique for detection and quantification of RNA expression. It is also used to detect the length polymorphism and imprinting status of the IGF2 gene [14,41]. There exists a CA repeat length polymorphism within exon-9 of the human IGF2 gene. The Ohlsson lab first applied this technique to discriminate two alleles derived from different parents to identify human *IGF2* imprinting. They prepared a RNA probe using T3 or T7 RNA polymerase with ^{32}P-UTP. The heterozygous sample was then identified by RPA with PCR product from genomic DNA. Next, RNA from the informative sample was directly subjected to RPA in order to check the imprinting status of *IGF2* [41]. The RPA method can be used to directly analyze RNA samples without the need of RT PCR and hence it excludes any possibilities of DNA contamination that can bias *IGF2* imprinting results. However, the method

also exposes researchers to isotopes. In addition, it can only be used to examine samples with CA repeat polymorphism in the IGF2 gene.

2.3. *Allele-Specific in Situ Hybridization* (*ASISH*) *technique*

The ASISH technique was developed based on RNA *in situ* hybridization performed by the Ohlsson lab [1, 40,42]. This method was based upon a single nucleotide difference within the *Apa I* polymorphic site within exon 9 of the IGF2 gene. Allelic-specific oligo DNA probes were designed to discriminate between expressed *IGF2* alleles with similar efficiency and specificity in the thin sections of formalin-fixed heterozygous tissues. Cui et al. decisively contributed to the development of the ASISH technique by improving the purification of 35S-labelled oligonucleotide DNA probes with longer hot poly A tails that greatly increased the sensitivity of the ASISH technique [9]. An important improvement of ASISH is that the technique can identify spatial and temporal allelic-specific expression patterns on a cellular level rather than relying on a sum of results of allelic expression patterns like other assays based on the total RNA. It may be applied in research for the timing of the establishment of functional imprinting during embryo development. The ASISH assay has helped to find the heterogenous imprinting pattern of *IGF2* in tumor tissues [42] as well as in determining the functional imprinting status of *H19* and *IGF2* at the cellular level in the development of the human placenta [1]. However, the main limitation of ASISH is that it is both time and labor consuming which are the reasons for its restricted use in laboratories.

2.4. *Other methods*

In addition to the methods mentioned above, there are several other techniques for the examination of *IGF2* imprinting. The simplest method consists of directly sequencing RT PCR products with *Apa I* polymorphism to determine the *IGF2* imprinting status according to its peak height at the SNP site. Clearly, direct sequencing is not quantitative for measuring allelic ratios while pyrosequencing, on the other hand, is a DNA sequencing technique based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides. The cascade starts with a nucleic acid polymerization reaction in which inorganic PPi is released due to nucleotide incorporation by polymerase. The released PPi is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light [50]. Pyrosequencing has been used for DNA genotyping [17]. We are currently in the process of applying this technique for the examination of the *IGF2* imprinting status. The pyrosequencing technique can precisely measure allelic ratios after RT PCR thus it is the fastest and most reliable method for the examination of *IGF2* imprinting although it may be limited by the expense of the pyrosequencing equipment. It is based on these techniques that the abnormal imprinting of *IGF2* was discovered in many human cancers.

3. LOI of *IGF2* in Childhood Tumors

Loss of imprinting of *IGF2* is the abnormal epigenetic activation of the normally silent maternal allele. *IGF2* was the first imprinted gene discovered to have LOI in several childhood tumors associated with Beckwith-Wiedemann syndrome (BWS) such as Wilms' tumor, hepatoblastoma, rhabdomyosarcoma, etc.

3.1. *Wilms' tumor*

Wilms' tumor shows a uniform worldwide incidence of 1:10,000, accounting for 6% of childhood cancers. It is also the most common malignant renal tumor with a peak incidence between 2 and 5 years old. Wilms' tumor usually occurs sporadically, but a low incidence ($\sim$1%) of familial cases has been reported [3,4]. Even though it has been suggested that the formation of several cancers might be related to genetic and epigenetic abnormalities, Wilms' tumor is currently the best explored. Rainier et al. [46] first found that 10 out of 13 informative Wilms' tumor showed LOI of *IGF2* (77%) while matched normal kidney tissues maintained normal imprinting. In the same issue of *Nature*, Ogawa et al. reported similar result that out of 6 informative Wilms' tumors, 4 showed LOI of *IGF2* (66%) [39]. Later, LOI of *IGF2* was continually confirmed and it was further uncovered that LOI of *IGF2* in Wilms' tumors is linked to the abnormal methylation of *H19-IGF2* locus and *H19* silencing in Wilms' tumor [8, 34,52,54,57,63]. Later, Ravenel et al. reported that LOI of *IGF2* was associated with a 2.2-fold increase in *IGF2* expression and children with LOI of *IGF2* in

their Wilms' tumors were statistically older at diagnosis than those whose tumors displayed normal imprinting. These results demonstrate the first evidence of LOI giving rise to the double dosage of expression of *IGF2* [48].

3.2. Rhabdomyosarcoma

Rhabdomyosarcoma is the second children's tumor identified to show LOI of *IGF2*. Like Wilms' tumor, Rhabdomyosarcoma is also an embryonal tumor associated with BWS and is the most common soft-tissue sarcoma in those children who often displayed the over-expression of *IGF2*. Zhan et al. found that out of 7 informative rhabdomyosarcoma cases, 5 tumors and 1 cell line showed LOI of *IGF2* and suggested that LOI might contribute to the over-expression of *IGF2* as well as play an important role in the onset of rhabdomyosarcoma [73].

3.3. Hepatoblastoma

Hepatoblastoma is another children's tumor related to BWS. Davies first examined 3 informative hepatoblastoma samples and indicated the absence of LOI of *IGF2* in hepatoblastoma [11]. Two laboratories later challenged the previous report and documented the presence of LOI of *IGF2* in hepatoblastomas [32, 45]. They also found that in contrast to Wilms' tumors, LOI of *IGF2* in hepatoblastoma is not linked to the down-regulation of *H19*. Obviously, unlike Wilms' tumor and rhabdomyosarcoma, hepatoblastomas has a low frequency of LOI of *IGF2*. Notably the IGF2 gene shows promoter-specific imprinting status and P2, P3 and P4 all display monoallelic activity in normal embryonic, neonatal and younger infants, whereas P1 is a non-imprinting promoter. From the embryonic period to 9 months of age, *IGF2* shows monoallelic expression due to the activation of P2, P3 and P4 but afterwards the gene displays biallelic expression due to the activation of P1 in livers [14]. Therefore, it is important to exclude the possibility of P1 activation when identifying LOI of *IGF2*. Li et al. investigated three hepatoblastomas derived from patients from 9 months to 3 years of age and found that there is a down-regulation of the P1 promoter while the P2, P3 promoters were up regulated in the tumor compared to normal liver. One of three cases showed LOI of *IGF2* in the tumor tissue [27]. Later, LOI of *IGF2* in hepatoblastoma was further confirmed by various other laboratories [19,22].

4. LOI of *IGF2* in adult tumors

The aberrant imprinting of *IGF2* occurs not only in embryonic tumors, but also frequently in a majority of adult tumors including prostate cancer, breast cancer, lung cancer, colon cancer, live cancer, etc. Elkin et al. first reported to find LOI of *IGF2* in bladder cancer despite the fact that they had only examined two informative samples [15]. Later, Jarrard et al. examined *IGF2* imprinting status in prostate cancer, a common cancer for men, and found that as high as 83% (10/12) of these tumors show LOI [21]. LOI of *IGF2* was also found in testicular tumor and 56% of these tumors displayed LOI [36]. Furthermore, aberrant imprinting was reported in women tumors as well, including breast cancer, ovarian cancer, and cervical carcinoma. Yballe et al. reported that 2 out of 17 breast tumors to have LOI [71] and Douc-Rasy et al. found abnormal imprinting of *IGF2* in 50% (5/10) of cervical tumors [13]. In addition, LOI of *IGF2* was reported repeatedly in ovarian cancer, another common cancer for women. Kim et al. found that as high as 55% (6/11) of these cancers displayed LOI [22]. Lung cancer and colon cancer, two of the most common cancers in the world for both males and females, are also associated with the imprinting disruption of *IGF2* with 25% (5/20) and 27% (6/22), respectively [5,68]. Cui et al reported that LOI of *IGF2* occurred in 44% of informative colorectal cancers that are linked to microsatellite instability [7]. Later, another group confirmed by reporting that 42% of sporadic colon cancers were related to LOI of *IGF2* [35]. Other laboratories have also reported variable rates of LOI, ranging from 33% to 87% [56, 74]. Consistent LOI of *IGF2* was also observed in lung cancer [26]; Kohda et al. later confirmed that 47% of these cases demonstrated LOI of *IGF2* [25]. Aberrant imprinting of *IGF2* was also found in human blood malignance. Wu et al. found that all of the 12 acute myeloid leukemia (AML) examined showed LOI of *IGF2* [66]. Furthermore, two other groups demonstrated LOI of *IGF2* in 72% (8/11) of AML and 54% (24/44) of acute lymphoblastic leukemia (ALL), respectively [29,62]. LOI of *IGF2* was found in various other adult tumors (see Table 1).

5. LOI of *IGF2* as a potential marker of risk of human cancers

Even though it has been found that the aberrant imprinting or LOI of *IGF2* is linked to many types of

Table 1
LOI of *IGF2* in adult cancers

Types of tumor	Frequency of *IGF2* LOI	References
Lung cancer	7/15 (46.6%)	Kohda et al., 2001 [25]
Hepatocellular carcinoma (HCC)	1/5 (20%)	Li et al., 1997 [28]
	6/7 (85%)	Kim et al., 1997 [23]
Breast cancer	2/17 (12%)	Yballe et al., 1996 [71]
	3/4 (75%)	Van Roozendaal et al., 1998 [61]
Colorectal cancer	12/27 (44%)	Cui et al., 1998 [7]
	4/12 (33%)	Takano et al.,2000 [56]
	14/16 (87.5%)	Zhang et al., 2003 [74]
Acute myeloid leukemia (AML)	12/12 (100%)	Wu et al., 1997 [66]
Acute lymphoblastic leukemia (ALL)	8/11 (72%)	Liu et al., 2000 [29]
Chronic myelogenous leukemia (CML)	24/44 (54%)	Vomerk et al., 2003 [62]
	7/12 (58%)	Randhawa et al.,1998 [47]
Ovarian cancer	0/11 (0%)	Yun et al., 1996 [72]
	6/11 (55%)	Jim et al., 1998 [22]
	5/20 (25%)	Chen et al., 2000 [5]
	6/22 (27%)	Xiong et al., 2002 [68]
Prostate cancer	10/12 (83%)	Jarrard et al., 1995 [21]
Bladder carcinoma	1/2 (50%)	Elkin et al., 1995 [15]
Cervical carcinoma	5/10 (50%)	Douc-Rasy et al., 1996 [13]
Esophageal cancer	7/13 (54%)	Mori et al., 1996 [33]
Glioma	8/14 (57%)	Uyeno et al., 1996 [60]
Testicular tumor	5/9 (55%)	Nonomura et al., 1997 [36]
Renal cell carcinoma	9/16 (56%)	Nonomura et al., 1997 [37]
	7/14 (50%)	Oda et al., 1998 [38]
Gynecologic tumor	5/24 (21%)	Yaginuma et al., 1997 [70]
Gastric adenocarcinoma	10/29 (34.5%)	Wu et al., 1997 [67]
Uterine leiomyoma	0/15 (0%)	Rainho et al., 1999 [44]
Pancreatic cancer	6/10 (60%)	Micha et al., 1999 [31]
Head and neck squamous carcinoma	11/27 (40.7%)	El-naggar et al., 1999 [16]
	2/10 (20%)	Rainho et al., 2001 [43]
Osteosarcoma	7/28 (25%)	Ulaner et al., 2003 [59]
Gestational trophoblastic tumors	7/18 (39%)	Kim et al., 2003 [24]
Juvenile nasopharyngeal angiofibromas	4/8 (50%)	Coutinho-Camillo et al., 2003 [6]
Choriocarcinoma	6/10 (60%)	Arima et al., 1997 [2]

human cancer, the most interesting discovery reveals the constitutional LOI in the normal tissues of cancer patients including matched normal tissues and peripheral blood lymphocytes. Although the exact mechanism by which aberrant imprinting leads to cancer is not yet known, it is believed that LOI of *IGF2* may be a potential biomarker for the risk of various cancers. When Cui *et al* examined the imprinting status of *IGF2* in colorectal cancer patients, they observed that LOI of *IGF2* existed not only in cancer tissues but also in the matched normal colonic mucosa and peripheral blood cells of patients with LOI [7]. Surprisingly, they also found LOI of *IGF2* in about 10% of colon mucosa and blood samples from the normal control group and thus first suggested that LOI might identify an important subset of the population with cancer or at risk of developing cancer. Next, a pilot study was conducted in which 172 patients at a colonoscopy clinic were evaluated and blood lymphocytes and colon biopsies were assayed. A significant association was reported between LOI of patients with family history or personal history of colorectal cancer, suggesting LOI of *IGF2* might be a valuable predictive biomarker for an individual's risk for colon cancer [10]. This interesting result was further confirmed by another colonoscopy screen study. Woodson et al. reported that 27.5% (11/40) of participants had LOI of *IGF2* in their normal colonic mucosa tissues and LOI was associated with a fivefold increased risk of adenoma formation in women [65].

In addition to its link to colon cancer, LOI of *IGF2* has also been investigated for an association with the risk of developing other malignancies. Vorwerk *et al* observed that 20% cord blood samples and 14% mononuclear cell (MNC) showed connection to LOI of *IGF2* when 50% of LOI was found in acute lymphoblastic leukemia (ALL) [62]. Unfortunately, the authors did not follow up their investigation and thus could not link LOI to the risk of the disease. In BWS, a children's disease predisposing many to childhood cancers, LOI of *IGF2* might be the best explanation for

the reason why aberrant imprinting is linked to tumorigenesis. Weksberg et al. were able to demonstrate the existence of LOI of *IGF2* in BWS fibroblast cells [64]. LOI may be a constitutional epigenetic aberrance that can occur early in normal tissue and blood cells and may predispose to the development of malignancies. Recently, a mouse model involving LOI of *IGF2* confirmed that LOI in mice increased the *IGF2* expression level and promoted the development of intestinal tumor, suggesting that the altered maturation of non-neoplastic tissue might be one mechanism through which epigenetic changes can affect cancer risk [51]. Based on experimental data, Feinberg et al. recently suggested an epigenetic progenitor model for cancer. They proposed that various cancers might have a common basis that is grounded in a polyclonal epigenetic disruption of stem/progenitor cells, mediated by "tumor-progenitor genes". Thus, non-neoplastic but epigenetically disrupted stem/progenitor cells might be a crucial target for cancer risk assessment and prevention [18].

Although LOI of *IGF2* is known to be associated with carcinogenesis, its mechanism is still uncertain. It has been reported that altered DNA methylation may link to LOI of *IGF2*. Wilms' tumors with LOI of *IGF2* indicated DNA hypermethylation in *H19* DMR [8,34, 52] while Colorectal cancers with LOI are linked to hypomethylation of *IGF2* DMR. Although it has been reported that knocking out DNA methyltransferases lead to both reduced DNA methylation and LOI of *IGF2* in human cell line [49], and mutation in DNMT3b gene, linked to hypomethylation in ICF syndrome [69], functional mutations could not be found in both Wilms' tumors and colorectal cancers with LOI of *IGF2* [10], thus suggesting DNA methyltransferases are not necessarily important in the maintenance of the normal imprinting of *IGF2*. A recent nutritional experiment showed that methyl donor-deficient diet induced LOI of *Igf2* in weaning mice, suggesting that childhood diet could contribute to *IGF2* LOI [63]. The mechanisms of LOI of *IGF2* and its relationship with tumorigenesis will become one of the most important research topics in the future.

6. Conclusion

LOI is an epigenetic disruption with a much higher incidence rate than conventional genetic mutations in human cancers. LOI of *IGF2* is involved in nearly all types of human cancers, including most of both childhood and adult cancers. It is well known that LOI of *IGF2* occurs not only in cancer cells, but also in matched normal tissue cells as well as the peripheral blood cells of cancer patients, suggesting LOI of *IGF2* to be an early epigenetic lesion that could predispose patients to the development of tumors. Furthermore, in contrast to conventional genetic mutations, this epigenetic lesion is generally considered reversible. Thus, research on the detection of LOI in human cancer patients will be of significant importance to the early diagnosis, prevention and even effective treatment of human cancers. It will also be very helpful in revealing the molecular mechanisms for the development of human cancers. Thus, future direction in research should focus on the search for a molecular epigenetic marker based on the DNA level, such as aberrant methylation corresponding to LOI on the RNA Level.

References

[1] G.I. Adam, H. Cui, S.J. Miller, F. Flam and R. Ohlsson, Allele-specific in situ hybridization (ASISH) analysis: a novel technique which resolves differential allelic usage of H19 within the same cell lineage during human placental development, *Development* **122** (1996), 839–847.

[2] T. Arima, T. Matsuda, N. Takagi and N. Wake, Association of IGF2 and H19 imprinting with choriocarcinoma development, *Cancer Genet Cytogenet* **93** (1997), 39–47.

[3] N.E. Breslow and J.B. Beckwith, Epidemiological features of Wilms' tumor: results of the National Wilms' Tumor Study, *J Natl Cancer Inst* **68** (1982), 429–436.

[4] N.E. Breslow, P.A. Norkool, A. Olshan, A. Evans and G.J. D'Angio, Second malignant neoplasms in survivors of Wilms' tumor: a report from the National Wilms' Tumor Study, *J Natl Cancer Inst* **80** (1988), 592–595.

[5] C.L. Chen, S.M. Ip, D. Cheng, L.C. Wong and H.Y. Ngan, Loss of imprinting of the IGF-II and H19 genes in epithelial ovarian cancer, *Clin Cancer Res* **6** (2000), 474–479.

[6] C.M. Coutinho-Camillo, M.M. Brentani, O. Butugan, H. Torloni and M.A. Nagai, Relaxation of imprinting of IGFII gene in juvenile nasopharyngeal angiofibromas, *Diagn Mol Pathol* **12** (2003), 57–62.

[7] H. Cui, I.L. Horon, R. Ohlsson, S.R. Hamilton and A.P. Feinberg, Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability, *Nat Med* **4** (1998), 1276–1280.

[8] H. Cui, E.L. Niemitz, J.D. Ravenel, P. Onyango, S.A. Brandenburg, V.V. Lobanenkov and A.P. Feinberg, Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutations of CTCF or its binding site, *Cancer Res* **61** (2001), 4947–4950.

[9] H. Cui and R. Ohlsson, An improved protocol for purification of 35S-labelled oligonucleotide DNA probes for in situ hybridization applications, *Trends Genet* **12** (1996), 506–507.

[10] H. Cui, P. Onyango, S. Brandenburg, Y. Wu, C.L. Hsieh and A.P. Feinberg, Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2, *Cancer Res* **62** (2002), 6442–6446.

[11] S.M. Davies, Maintenance of genomic imprinting at the IGF2 locus in hepatoblastoma, *Cancer Res* **53** (1993), 4781–4783.

[12] T.M. DeChiara, E.J. Robertson and A. Efstratiadis, Parental imprinting of the mouse insulin-like growth factor II gene, *Cell* **64** (1991), 849–859.
[13] S. Douc-Rasy, M. Barrois, S. Fogel, J.C. Ahomadegbe, D. Stehelin, J. Coll and G. Riou, High incidence of loss of heterozygosity and abnormal imprinting of H19 and IGF2 genes in invasive cervical carcinomas. Uncoupling of H19 and IGF2 expression and biallelic hypomethylation of H19, *Oncogene* **12** (1996), 423–430.
[14] T.J. Ekstrom, H. Cui, X. Li and R. Ohlsson, Promoter-specific IGF2 imprinting status and its plasticity during human liver development, *Development* **121** (1995), 309–316.
[15] M. Elkin, A. Shevelev, E. Schulze, M. Tykocinsky, M. Cooper, I. Ariel, D. Pode, E. Kopf, N. de Groot and A. Hochberg, The expression of the imprinted H19 and IGF-2 genes in human bladder carcinoma, *FEBS Lett* **374** (1995), 57–61.
[16] A.K. el-Naggar, S. Lai, S.A. Tucker, G.L. Clayman, H. Goepfert, W.K. Hong and V. Huff, Frequent loss of imprinting at the IGF2 and H19 genes in head and neck squamous carcinoma, *Oncogene* **18** (1999), 7063–7069.
[17] H. Fakhrai-Rad, N. Pourmand and M. Ronaghi, Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms, *Hum Mutat* **19** (2002), 479–485.
[18] A.P. Feinberg, R. Ohlsson and S. Henikoff, The epigenetic progenitor origin of human cancer, *Nat Rev Genet* **7** (2006), 21–33.
[19] R. Fukuzawa, A. Umezawa, K. Ochi, F. Urano, H. Ikeda and J. Hata, High frequency of inactivation of the imprinted H19 gene in "sporadic" hepatoblastoma, *Int J Cancer* **82** (1999), 490–497.
[20] C.F. Graham, The production of parthenogenetic mammalian embryos and their use in biological research, *Biol Rev Camb Philos Soc* **49** (1974), 399–424.
[21] D.F. Jarrard, M.J. Bussemakers, G.S. Bova and W.B. Isaacs, Regional loss of imprinting of the insulin-like growth factor II gene occurs in human prostate tissues, *Clin Cancer Res* **1** (1995), 1471–1478.
[22] H.T. Kim, B.H. Choi, N. Niikawa, T.S. Lee and S.I. Chang, Frequent loss of imprinting of the H19 and IGF-II genes in ovarian tumors, *Am J Med Genet* **80** (1998), 391–395.
[23] K.S. Kim and Y.I. Lee, Biallelic expression of the H19 and IGF2 genes in hepatocellular carcinoma, *Cancer Lett* **119** (1997), 143–148.
[24] S.J. Kim, S.E. Park, C. Lee, S.Y. Lee, I.H. Kim, H.J. An and Y.K. Oh, Altered imprinting, promoter usage, and expression of insulin-like growth factor-II gene in gestational trophoblastic diseases, *Gynecol Oncol* **88** (2003), 411–418.
[25] M. Kohda, H. Hoshiya, M. Katoh, I. Tanaka, R. Masuda, T. Takemura, M. Fujiwara and M. Oshimura, Frequent loss of imprinting of IGF2 and MEST in lung adenocarcinoma, *Mol Carcinog* **31** (2001), 184–191.
[26] M. Kondo and T. Takahashi, Altered genomic imprinting in the IGF2 and H19 genes in human lung cancer, *Nippon Rinsho* **54** (1996), 492–496.
[27] X. Li, G. Adam, H. Cui, B. Sandstedt, R. Ohlsson and T.J. Ekstrom, Expression, promoter usage and parental imprinting status of insulin-like growth factor II (IGF2) in human hepatoblastoma: uncoupling of IGF2 and H19 imprinting, *Oncogene* **11** (1995), 221–229.
[28] X. Li, Z. Nong, C. Ekstrom, E. Larsson, H. Nordlinder, W.J. Hofmann, C. Trautwein, M. Odenthal, H.P. Dienes, T.J. Ekstrom and P. Schirmacher, Disrupted IGF2 promoter control by silencing of promoter P1 in human hepatocellular carcinoma, *Cancer Res* **57** (1997), 2048–2054.
[29] Y. Liu, B. Yang, H. Zhu and J. Wang, Loss of imprinting of insulin-like growth factor II gene in acute myeloid leukemia, *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* **17** (2000), 39–41.
[30] J. McGrath and D. Solter, Completion of mouse embryogenesis requires both the maternal and paternal genomes, *Cell* **37** (1984), 179–183.
[31] A.E. Micha, S. Hahnel, H. Friess, M.W. Buchler, G. Adler and T.M. Gress, Genomic imprinting of IGF-II and H19 in adult human pancreatic tissues, *Digestion* **60** (1999), 477–483.
[32] M. Montagna, C. Menin, L. Chieco-Bianchi and E. D'Andrea, Occasional loss of constitutive heterozygosity at 11p15.5 and imprinting relaxation of the IGFII maternal allele in hepatoblastoma, *J Cancer Res Clin Oncol* **120** (1994), 732–736.
[33] M. Mori, H. Inoue, T. Shiraishi, K. Mimori, K. Shibuta, H. Nakashima, K. Mafune, Y. Tanaka, H. Ueo, G.F. Barnard, K. Sugimachi and T. Akiyoshi, Relaxation of insulin-like growth factor 2 gene imprinting in esophageal cancer, *Int J Cancer* **68** (1996), 441–446.
[34] T. Moulton, T. Crenshaw, Y. Hao, J. Moosikasuwan, N. Lin, F. Dembitzer, T. Hensle, L. Weiss, L. McMorrow, T. Loew et al., Epigenetic lesions at the H19 locus in Wilms' tumour patients, *Nat Genet* **7** (1994), 440–447.
[35] S. Nishihara, T. Hayashida, K. Mitsuya, T.C. Schulz, M. Ikeguchi, N. Kaibara and M. Oshimura, Multipoint imprinting analysis in sporadic colorectal cancers with and without microsatellite instability, *Int J Oncol* **17** (2000), 317–322.
[36] N. Nonomura, T. Miki, K. Nishimura, N. Kanno, Y. Kojima and A. Okuyama, Altered imprinting of the H19 and insulin-like growth factor II genes in testicular tumors, *J Urol* **157** (1997), 1977–1979.
[37] N. Nonomura, K. Nishimura, T. Miki, N. Kanno, Y. Kojima, M. Yokoyama and A. Okuyama, Loss of imprinting of the insulin-like growth factor II gene in renal cell carcinoma, *Cancer Res* **57** (1997), 2575–2577.
[38] H. Oda, H. Kume, Y. Shimizu, T. Inoue and T. Ishikawa, Loss of imprinting of igf2 in renal-cell carcinomas, *Int J Cancer* **75** (1998), 343–346.
[39] O. Ogawa, M.R. Eccles, J. Szeto, L.A. McNoe, K. Yun, M.A. Maw, P.J. Smith and A.E. Reeve, Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour, *Nature* **362** (1993), 749–751.
[40] R. Ohlsson, H. Cui, L. He, S. Pfeifer, H. Malmikumpu, S. Jiang, A.P. Feinberg and F. Hedborg, Mosaic allelic insulin-like growth factor 2 expression patterns reveal a link between Wilms' tumorigenesis and epigenetic heterogeneity, *Cancer Res* **59** (1999), 3889–3892.
[41] R. Ohlsson, A. Nystrom, S. Pfeifer-Ohlsson, V. Tohonen, F. Hedborg, P. Schofield, F. Flam and T.J. Ekstrom, IGF2 is parentally imprinted during human embryogenesis and in the Beckwith-Wiedemann syndrome, *Nat Genet* **4** (1993), 94–97.
[42] R. Ohlsson, K. Svensson, H. Cui, H. Malmikumpu and G. Adam, Allele-Specific In SituHybridization (ASISH), *Methods Mol Biol* **181** (2001), 153–167.
[43] C.A. Rainho, L.P. Kowalski and S.R. Rogatto, Loss of imprinting and loss of heterozygosity on 11p15.5 in head and neck squamous cell carcinomas, *Head Neck* **23** (2001), 851–859.
[44] C.A. Rainho, A. Pontes and S.R. Rogatto, Expression and imprinting of insulin-like growth factor II (IGF2) and H19 genes in uterine leiomyomas, *Gynecol Oncol* **74** (1999), 375–380.
[45] S. Rainier, C.J. Dobry and A.P. Feinberg, Loss of imprinting in hepatoblastoma, *Cancer Res* **55** (1995), 1836–1838.

[46] S. Rainier, L.A. Johnson, C.J. Dobry, A.J. Ping, P.E. Grundy and A.P. Feinberg, Relaxation of imprinted genes in human cancer, *Nature* **362** (1993), 747–749.
[47] G.S. Randhawa, H. Cui, J.A. Barletta, L.Z. Strichman-Almashanu, M. Talpaz, H. Kantarjian, A.B. Deisseroth, R.C. Champlin and A.P. Feinberg, Loss of imprinting in disease progression in chronic myelogenous leukemia, *Blood* **91** (1998), 3144–3147.
[48] J.D. Ravenel, K.W. Broman, E.J. Perlman, E.L. Niemitz, T.M. Jayawardena, D.W. Bell, D.A. Haber, H. Uejima and A.P. Feinberg, Loss of imprinting of insulin-like growth factor-II (IGF2) gene in distinguishing specific biologic subtypes of Wilms tumor, *J Natl Cancer Inst* **93** (2001), 1698–1703.
[49] I. Rhee, K.E. Bachman, B.H. Park, K.W. Jair, R.W. Yen, K.E. Schuebel, H. Cui, A.P. Feinberg, C. Lengauer, K.W. Kinzler, S.B. Baylin and B. Vogelstein, DNMT1 and DNMT3b cooperate to silence genes in human cancer cells, *Nature* **416** (2002), 552–556.
[50] M. Ronaghi, Pyrosequencing sheds light on DNA sequencing, *Genome Res* **11** (2001), 3–11.
[51] T. Sakatani, A. Kaneda, C.A. Iacobuzio-Donahue, M.G. Carter, S. de Boom Witzel, H. Okano, M.S. Ko, R. Ohlsson, D.L. Longo and A.P. Feinberg, Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice, *Science* **307** (2005), 1976–1978.
[52] M.J. Steenman, S. Rainier, C.J. Dobry, P. Grundy, I.L. Horon and A.P. Feinberg, Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumour, *Nat Genet* **7** (1994), 433–439.
[53] L.C. Stevens, Totipotent cells of parthenogenetic origin in a chimaeric mouse, *Nature* **276** (1978), 266–267.
[54] M.J. Sullivan, T. Taniguchi, A. Jhee, N. Kerr and A.E. Reeve, Relaxation of IGF2 imprinting in Wilms tumours associated with specific changes in IGF2 methylation, *Oncogene* **18** (1999), 7527–7534.
[55] M.A. Surani, S.C. Barton and M.L. Norris, Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis, *Nature* **308** (1984), 548–550.
[56] Y. Takano, G. Shiota and H. Kawasaki, Analysis of genomic imprinting of insulin-like growth factor 2 in colorectal cancer, *Oncology* **59** (2000), 210–216.
[57] T. Taniguchi, M.J. Sullivan, O. Ogawa and A.E. Reeve, Epigenetic changes encompassing the IGF2/H19 locus associated with relaxation of IGF2 imprinting and silencing of H19 in Wilms tumor, *Proc Natl Acad Sci USA* **92** (1995), 2159–2163.
[58] H. Uejima, M.P. Lee, H. Cui and A.P. Feinberg, Hot-stop PCR: a simple and general assay for linear quantitation of allele ratios, *Nat Genet* **25** (2000), 375–376.
[59] G.A. Ulaner, T.H. Vu, T. Li, J.F. Hu, X.M. Yao, Y. Yang, R. Gorlick, P. Meyers, J. Healey, M. Ladanyi and A.R. Hoffman, Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site, *Hum Mol Genet* **12** (2003), 535–549.
[60] S. Uyeno, Y. Aoki, M. Nata, K. Sagisaka, T. Kayama, T. Yoshimoto and T. Ono, IGF2 but not H19 shows loss of imprinting in human glioma, *Cancer Res* **56** (1996), 5356–5359.
[61] C.E. van Roozendaal, A.J. Gillis, J.G. Klijn, B. van Ooijen, C.J. Claassen, A.M. Eggermont, S.C. Henzen-Logmans, J.W. Oosterhuis, J.A. Foekens and L.H. Looijenga, Loss of imprinting of IGF2 and not H19 in breast cancer, adjacent normal tissue and derived fibroblast cultures, *FEBS Lett* **437** (1998), 107–111.
[62] P. Vorwerk, H. Wex, C. Bessert, B. Hohmann, U. Schmidt and U. Mittler, Loss of imprinting of IGF-II gene in children with acute lymphoblastic leukemia, *Leuk Res* **27** (2003), 807–812.
[63] R.A. Waterland, J.R. Lin, C.A. Smith and R.L. Jirtle, Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus, *Hum Mol Genet* **15** (2006), 705–716.
[64] R. Weksberg, D.R. Shen, Y.L. Fei, Q.L. Song and J. Squire, Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome, *Nat Genet* **5** (1993), 143–150.
[65] K. Woodson, A. Flood, L. Green, J.A. Tangrea, J. Hanson, B. Cash, A. Schatzkin and P. Schoenfeld, Loss of insulin-like growth factor-II imprinting and the presence of screen-detected colorectal adenomas in women, *J Natl Cancer Inst* **96** (2004), 407–410.
[66] H.K. Wu, R. Weksberg, M.D. Minden and J.A. Squire, Loss of imprinting of human insulin-like growth factor II gene, IGF2, in acute myeloid leukemia, *Biochem Biophys Res Commun* **231** (1997), 466–472.
[67] M.S. Wu, H.P. Wang, C.C. Lin, J.C. Sheu, C.T. Shun, W.J. Lee and J.T. Lin, Loss of imprinting and overexpression of IGF2 gene in gastric adenocarcinoma, *Cancer Lett* **120** (1997), 9–14.
[68] Y. Xiong, Y. Sun and H. Li, Different imprinting status of IGF-2 in epithelial ovarian tumors, *J Huazhong Univ Sci Technolog Med Sci* **22** (2002), 255–256.
[69] G.L. Xu, T.H. Bestor, D. Bourc'his, C.L. Hsieh, N. Tommerup, M. Bugge, M. Hulten, X. Qu, J.J. Russo and E. Viegas-Pequignot, Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene, *Nature* **402** (1999), 187–191.
[70] Y. Yaginuma, K. Nishiwaki, S. Kitamura, H. Hayashi, K. Sengoku and M. Ishikawa, Relaxation of insulin-like growth factor-II gene imprinting in human gynecologic tumors, *Oncology* **54** (1997), 502–507.
[71] C.M. Yballe, T.H. Vu and A.R. Hoffman, Imprinting and expression of insulin-like growth factor-II and H19 in normal breast tissue and breast tumor, *J Clin Endocrinol Metab* **81** (1996), 1607–1612.
[72] K. Yun, M. Fukumoto and Y. Jinno, Monoallelic expression of the insulin-like growth factor-2 gene in ovarian cancer, *Am J Pathol* **148** (1996), 1081–1087.
[73] S. Zhan, D.N. Shapiro and L.J. Helman, Activation of an imprinted allele of the insulin-like growth factor II gene implicated in rhabdomyosarcoma, *J Clin Invest* **94** (1994), 445–448.
[74] F.R. Zhang, X.B. He, Y.H. Yang and W. Xie, The expression and imprinting status of insulin-like growth factor 2 gene in colorectal cancer, *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* **20** (2003), 31–34.