Loss of imprinting of IGF2 and not H19 in breast cancer, adjacent normal tissue and derived fibroblast cultures

C.E.P. van Roozendaal[1,a], A.J.M. Gillis[b], J.G.M. Klijn[a], B. van Ooijen[c], C.J.C. Claassen[a], A.M.M. Eggermont[c], S.C. Henzen-Logmans[d], J.W. Oosterhuis[b], J.A. Foekens[a,*], L.H.J. Looijenga[b]

[a] *Division of Endocrine Oncology (Department of Medical Oncology), Daniel den Hoed Cancer Center/Academic Hospital, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands*

[b] *Laboratory for Experimental Patho-Oncology, Daniel den Hoed Cancer Center/Academic Hospital, Rotterdam, The Netherlands*

[c] *Department of Surgical Oncology, Daniel den Hoed Cancer Center/Academic Hospital, Rotterdam, The Netherlands*

[d] *Department of Pathology, Daniel den Hoed Cancer Center/Academic Hospital, Rotterdam, The Netherlands*

Received 9 August 1998

Abstract Insulin-like growth factors are involved in the paracrine growth regulation of human breast tumor cells. IGF2 is imprinted in most tissues, and shows expression of the paternal allele only. To investigate whether disruption of this monoallelic IGF2 expression is involved in breast cancer development, a series of primary tumors and adjacent, histologically normal, breast tissue samples, as well as matched primary in vitro fibroblast cultures were studied. Biallelic expression (partial) of IGF2 was found in the majority of in vivo samples, and corresponding fibroblast cultures, while monoallelic expression was found in a normal breast sample. In contrast, H19, a closely apposed, but reciprocally imprinted gene, assumed to be regulated by a common control element, showed retention of monoallelic H19 expression in all in vivo and in the majority of in vitro samples. These data indicate that IGF2, but not H19, is prone to loss of imprinting in breast cancer.

Key words: Breast cancer; Stromal fibroblast; Insulin-like growth factor; Genomic imprinting

1. Introduction

A complex bi-directional interaction between malignant epithelial cells and surrounding stromal tissue appears to be crucial in the development and progression of breast cancer. In addition to the frequently detected genetic alterations in breast tumor cells [1], stromal cells within or immediately adjacent to the neoplastic site have been shown to display phenotypical changes [2,3]. Although a variety of different growth factors are probably involved [4], insulin-like growth factors (IGFs) secreted by fibroblasts have been shown to be, at least partly, responsible for the induced breast tumor cell proliferation in vitro [5]. Two different IGFs have been distinguished, i.e. IGF1 and IGF2, and numerous studies have shown the involvement of these two factors in growth regulation of breast cancer and other malignancies [6,7].

Interestingly, IGF2 has been shown to be imprinted, an epigenetic phenomenon describing the differential expression of alleles based on their parental origin ([8,9] for review), and this gene is suggested to be part of an imprinted domain together with the H19 gene [10]. While IGF2 is preferentially expressed in most tissues from the paternal allele [11], H19 is expressed only from the maternal allele [12]. Regulation of IGF2 and H19 expression has been suggested to be closely linked and to involve a common enhancer element [10,13]. Coordinated allele-specific expression of both IGF2 and H19 appears to be important for normal embryonal development ([14] for review). Furthermore, loss of imprinting (LOI) and/or structural chromosomal alteration in the IGF2/H19 locus has been suggested to correlate with development of a number of both childhood and adult tumors, for example, sporadic Wilms' tumors, bladder, cervical, lung and prostate carcinomas and testicular germ cell tumors [9,15]. In addition, constitutional loss of IGF2 imprinting has been demonstrated in in vitro cultures of skin fibroblast derived from Beckwith-Wiedemann syndrome patients, which are prone to develop cancer [16].

In this study we investigated the presence of LOI of IGF2 and H19 in breast cancer. For this purpose we analyzed the allele-specific expression of these genes by a reverse transcription-polymerase chain reaction (RT-PCR) approach, in a series of matched primary tumor and adjacent, histologically normal, breast tissue samples as well as primary in vitro fibroblast cultures established from these tissues.

2. Materials and methods

2.1. Cell culture

Primary fibroblast cultures were established from breast carcinomas and adjacent histologically normal breast tissue ($n = 8$), and propagated as described previously [5].

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from subconfluent primary fibroblast cultures (at passage 5–8) and cryosections from corresponding frozen tissue using RNA STAT-60 according to the protocol of the supplier (Tel-Test 'B'). Parallel cryosections were used for histology. cDNA was synthesized using MMLV reverse transcriptase (Gibco-BRL, Breda, Netherlands).

2.3. IGF2 and H19 gene expression

The expression of the IGF2 and H19 genes, in both the in vitro and in vivo samples, was determined by PCR amplification of the generated cDNA, using gene specific primer combinations spanning intron sequences. The following primers were used: IGF2: IG-4a, 5′-CCTGGAGACGTACTGTGCTACC-3′ and IG-8, 5′-TGGGTAGAGCAATCAGGGGAC-3′ (annealing at 62°C); H19: HN-6, 5′-CTTTCATGTTGTGGGTTCTGGGA-3′ and HN-7, 5′-CCAGGTCTCCAGCTGGGGTG-3′ (annealing at 64°C). Negative controls were used in every PCR assay and included samples without reverse transcrip-

*Corresponding author. Fax: (31) (10) 4232 964.
E-mail: foekens@bidh.azr.nl

[1]Present address: Dept. of Cell Biology, KUN, Nijmegen, The Netherlands

PII: S0014-5793(98)01211-3

tase (RNA control), and samples where cDNA was omitted (water control).

2.4. Detection of IGF2 and H19 polymorphism

Genomic DNA was extracted from frozen tissue using standard techniques. The IGF2 allelic pattern for each patient was determined using the *Apa*I polymorphism located in the untranslated region of exon 9; primers: IG-for, 5′-CTTGGACTTTGAGTCAAATTGG-3′ and IG-5, 5′-GGGTCGTGCCAATTACATTTCAT-3′, annealing temp. 56°C [17]. The polymorphic sites *Avi*II or *Rsa*I, both located within exon 5 and only 40 bp apart, were analyzed for the H19 gene (primers: HN-19, 5′-TGACTGAGGAATCGGCTCTGGAAG-3′ and HN-20, 5′-CGGTCGGAGCTTCCAGACTAG-3′, annealing temp. 64°C). The polymerase chain reaction (PCR), subsequent digestion and analysis were performed as previously described [18].

2.5. Allele-specific gene expression

The allele-specific IGF2 and H19 expression of the informative samples was determined, in at least two independently isolated RNA preparations of each sample, using the primer combinations IG-for/IG-5 (IGF2) and HN19/HN20 (H19). To avoid interference of contaminating DNA in the allele-specific IGF2 expression analysis the RNA was DNase-treated, prior to the cDNA synthesis. Therefore, RNA was incubated with 20 units RNase-free DNase (Boehringer-Mannheim) for 30 min at 37°C. The samples were subsequently phenol/chloroform extracted and ethanol precipitated. Each resuspended RNA sample was evenly split and incubated with or without reverse transcriptase (RT) as describe above. In the subsequent PCR the additional negative (minus RT) control was included for each sample. Only those DNase-treated RNAs, irrefutably shown to be devoid of DNA, were used for the subsequent analysis of the IGF2 allele-specific expression. The obtained cDNA-PCR products were digested to completion using the required restriction enzyme and analyzed on a 2% Nusieve agarose ethidium bromide gel.

3. Results and discussion

The clinical and pathological data of the eight cases included in this study are summarized in Table 1. All patients were older than 35 years, with an average age of 54.4. All tumors were primary breast cancers, and surgically removed. Only patient #8 was pretreated with chemotherapy prior to surgery. Six cases were diagnosed as ductal carcinomas, and two (cases #2 and #4) as mixed, containing both ductal and lobular carcinoma elements. Three patients had a positive axillary lymph node status. The estrogen and progesterone receptor status varied between 0–546, and 0–218 fmol/mg protein, respectively.

All primary tumors and adjacent, histologically normal, breast tissues in vivo as well as in the derived in vitro fibroblast cultures showed expression using RT-PCR of both IGF2 and H19 (results not shown). Subsequently, we studied the imprinted status, i.e. mono-/biallelic expression, of these genes. Analysis of the H19-informative cases, using the polymorphic *Avi*II site, revealed monoallelic expression in all five primary tumor and all four available adjacent histologically normal tissues in vivo (see Fig. 1A and Table 2). In the three patients shown to be also informative for the *Rsa*I site, the monoallelic H19 expression in vivo was confirmed. Similarly, H19 imprinting was sustained in most of the in vitro fibroblast cultures. In one case (case #8), however, both the tumor and normal tissue derived from in vitro fibroblast cultures displayed biallelic H19 expression, although the corresponding in vivo tumor tissue showed monoallelic expression. In addition to the digestion analysis, the results were verified by direct sequencing. As shown in Fig. 1B, both the allele with the *Avi*II-restriction site (-TGCGCA-) and the digestion resistant polymorphic specific allelic sequence (-TGTGCA-) were detected. Because of the fact that the in vivo sample predominantly consisted of tumor cells, the monoallelic H19 expression detected in this sample could merely reflect the expression in tumor cells, rather than in the stromal fibroblasts. Indeed, H19 expression has been observed in both epithelial and stromal cells in breast tumor and adjacent normal tissue using in situ hybridization [19]. Although we cannot exclude a culture specific induced LOI, similar to an observation by Eversole-Cire et al. [20], the phenomenon of biallelic H19 expression in the fibroblast cultures is obviously not a general in vitro induced effect and thus indicates that the in vivo imprinting status is mostly maintained in vitro. The observed in vitro biallelic H19 expression could be related to the chemotherapeutic treatment this patient received before surgical removal of the cancer. Overall our data suggest that LOI of H19 is not a general phenomenon in the development and/or progression of breast cancer, confirming a recent study by Yballe et al. [21], in contrast to the finding in other cancers ([9,15] for review).

Our study has furthermore revealed an intriguing allelic switch of H19 expression in case #1 (see Fig. 1A and Table 2). While monoallelic expression of the *Avi*II digested allele was detected in the in vivo tumor tissue, only the undigested allele was shown to be expressed in the adjacent normal tissue and the fibroblast cultures in vitro. This suggests that the tumor cell population has switched H19 expression to the otherwise silent allele. Monoallelic expression of the normally silent H19 allele has recently also been observed in cervical carcinomas [22]. In normal adult cerebellum tissue a similar switch has been described by Zhang et al. [23]. In addition, their study revealed biallelic H19 expression in lung tissue, while in the other tissues monoallelic H19 expression was

Table 1
Summary of the clinical and pathological data of the breast tumor cases

Case	Age (years)	Histology (carcinoma)	Tumor size[b]	Nodal status	ER/PR[c]	Follow up
#1	51	Ductal	T1	−	76/218	Alive, relapse after 6 years
#2	66	Mixed[a]	T1	+	43/28	Alive, NED[d] after 6.5 years
#3	60	Ductal	T2	−	5/0	Dead of disease after 4 years
#4	53	Ductal	T2	−	37/5	Alive, relapse after 6 years
#5	42	Mixed	T2	−	0/17	Dead of disease after 4.5 years
#6	36	Ductal	T1	−	4/0	Dead of disease after 1.5 years
#7	79	Ductal	T1	+	546/6	Dead of disease after 2.5 years
#8	48	Ductal	T3/4	+	71/190	Alive, NED after 3.5 years

[a]Containing both ductal and lobular elements.
[b]T1 = < 2 cm, T2 = 2–5 cm, T3/4 = > 5 cm.
[c]Estrogen and progesterone receptor levels in fmol/mg protein.
[d]No evidence of disease.

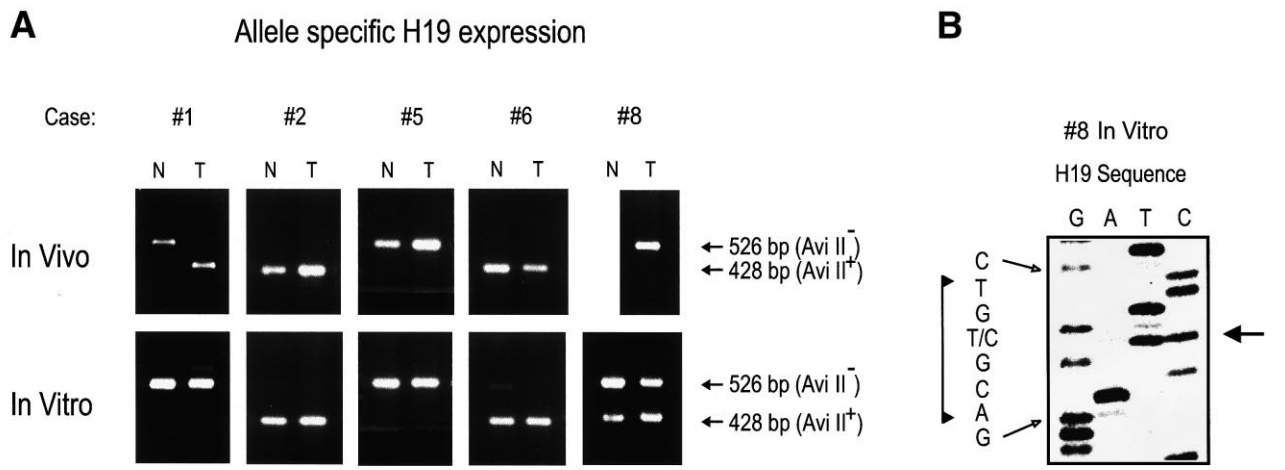

Fig. 1. A: Results of the allele-specific expression analysis of H19 as determined by RT-PCR in samples of breast tumor patients informative for the *Avi*II polymorphism. Shown are the *Avi*II restriction endonuclease digests of the amplification products (generated by the HN19/HN20 primers) from primary tumor tissues (T) and adjacent normal breast tissues (N) in vivo and the derived fibroblast in vitro cultures. Note the monoallelic expression of H19 in all samples, except the fibroblast cultures of case #8. B: Part of the H19 sequence, as obtained by direct sequencing of the RT-PCR product from the in vitro tumor sample from case #8. The arrow indicates the presence of amplification products representative for both alleles, demonstrated by identification of a thymidine (T) and a cytosine (C) within the *Avi*II restriction recognition site (-TGCGCA-).

detected. The observed variability of H19 allele-specific expression in this patient thus supports a previously suggested cell type or tissue specific regulatory mechanism.

In contrast to H19, our analysis of the IGF2 informative cases showed partial LOI in the majority of in vivo samples, derived not only from the tumor but also from the adjacent, histologically normal tissues (see Fig. 2A and Table 3), as revealed by the presence of both the *Apa*I-resistant A-allele and the *Apa*I-sensitive B-allele after digestion of the RT-PCR products. Remarkable, LOI appears to be confined to the IGF2 gene, as was especially illustrated by cases #5 and #6, which were also informative for H19 (see Fig. 1A and Table 2). In only one of the informative patients (case #4) monoallelic IGF2 expression was detected in the primary tumor and adjacent normal tissue. Interestingly, this patient was still alive after 6 years of diagnosis of the initial tumor, although a relapse was found, while the other three patients died of their disease within 4.5 years after initial diagnosis. To determine whether this is a significant observation requires a more extended analysis. However, the data suggest that expression of IGF2 is regulated in a similar fashion in both tissue sites. Interestingly, the allele with the restriction site (the B-allele) was most predominant, while an equal ratio of both alleles was observed in the respective DNA samples analyzed (results not shown). Variable levels of relaxation have also been described by others [24], probably reflecting heterogeneity of the samples under investigation. To confirm that the observed A-allele is genuinely present, the RT-PCR products were analyzed by direct sequencing. This indeed revealed the presence of the expected, *Apa*I-resistant (-GGACCC-) allelic sequence (not shown). In addition, the restriction resistant fragments, isolated from the gel after the initial digest analysis, were reamplified and *Apa*I digested. Again, as expected, these products remained undigested (results not shown) confirming the previous data. Unfortunately, the parental origin of the alleles could not be determined due to lack of parental DNA.

An IGF2 allele-specific expression pattern, similar to the in vivo samples, was observed for most corresponding in vitro samples (Fig. 2A and Table 3). As indicated for H19, this again suggests that the in vivo imprinting status is generally maintained in early passage primary fibroblast cultures. Although LOI in cultured cells can occur after multiple passages, maintenance of genomic imprinting in early passage

Table 2
Allele specific H19 expression in the informative primary breast tumor, corresponding adjacent histologically normal tissue, and matched fibroblast in vitro cultures

Case	Tissue	In vivo		In vitro	
		*Avi*II	*Rsa*I	*Avi*II	*Rsa*I
#1	Normal	A	B	A	B
	Tumor	B	A	A	B
#2	Normal	B	A	B	A
	Tumor	B	A	B	A
#5	Normal	A	B	A	B
	Tumor	A	B	A	B
#6	Normal	B	NI[a]	B	NI
	Tumor	B	NI	B	NI
#8	Normal	NA[b]	NA	A/B[c]	NI
	Tumor	A	NI	A/B	NI

[a]Not informative.
[b]Not available.
[c]Balanced expression of the A and B allele.

Table 3
Allele-specific IGF2 expression in the informative primary breast tumor, corresponding adjacent histologically normal tissue, and matched fibroblast in vitro cultures

Case	Tissue	In vivo	In vitro
#4	Normal	A	A
	Tumor	A	B
#5	Normal	aB[a]	aB
	Tumor	aB	A
#6	Normal	aB	aB
	Tumor	aB	aB
#7	Normal	NA[b]	AB[c]
	Tumor	aB	AB

[a]Expression of A-allele between 10–25% of total level.
[b]Not available.
[c]Balanced expression of the A and B allele.

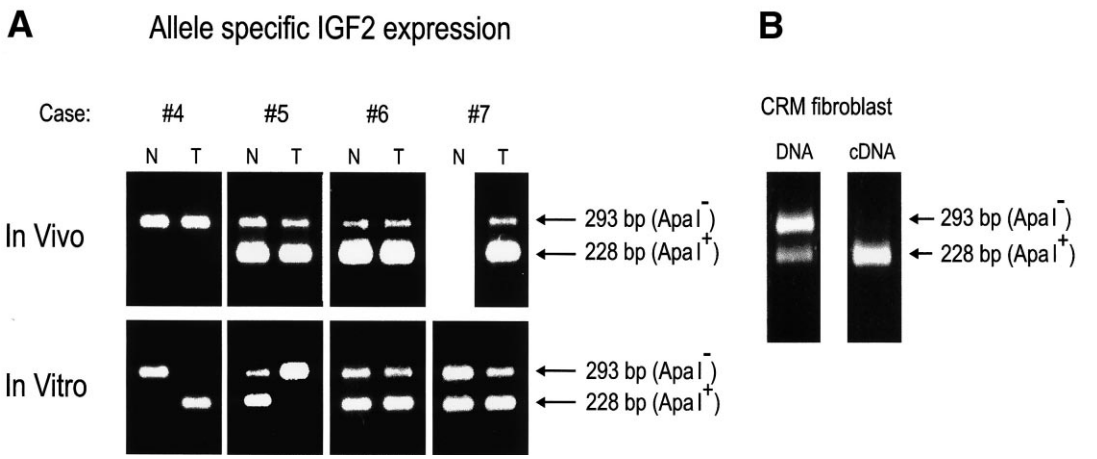

Fig. 2. A: Results of the allele-specific expression analysis of IGF2 as determined by RT-PCR in in vivo tissue samples and matched in vitro fibroblast cultures of breast tumor patients informative for the *Apa*I polymorphism. Shown are the *Apa*I restriction digest of the amplification products (generated by the IG-for/IG-5 primers) from primary tumor tissue (T) and adjacent normal breast tissue (N) in vivo and the derived in vitro fibroblast cultures. Note the (partial) biallelic expression in all cases, except case #4. B: Results of allele-specific IGF2 expression in an *Apa*I informative in vitro fibroblast culture established from a cosmetic reduction mammaplasty tissue sample (CRM = cosmetic reduction mammaplasty). Shown are the *Apa*I restriction digest of the PCR-amplified fibroblast DNA and cDNA products (generated by the IG-for/IG-5 primers). Note the monoallelic expression of the B allele.

cultures has previously been described [16,25]. In addition, monoallelic IGF2 expression was found, as expected, in a fibroblast culture derived from a cosmetic reduction mammoplasty (see Fig. 2B). Our data thus suggest that, in contrast to H19, LOI of IGF2 is involved in breast cancer development and/or progression. While relaxation of IGF2 imprinting in breast tumor, as shown here, has been reported by others [21,24,26], our study further indicates the presence of epigenetically modified cells in the adjacent normal tissue. Biallelic expression of IGF2 in not only the tumor cells but also the associated normal tissue has also been reported by others in breast [22], and in other tumor types, including those of the prostate and Wilms' tumor [17,27]. This indicates that besides phenotypical changes, as previously described [2,3], the stromal cells within, or immediately adjacent to the cancer also show changes in the allelic expression pattern of IGF2. This does not seem to be influenced by clinical and/or pathological parameters.

An intriguing result was obtained upon the IGF2 analysis of one of the tumor tissue derived fibroblast cultures (case #5). Although the original tissue in vivo was shown to express both alleles, the corresponding in vitro sample displayed monoallelic IGF2 expression. The in vivo preferentially expressed allele was shown to be fully silent in the in vitro sample. Furthermore, the allelic switch observed was shown to be confined to the IGF2 gene, because the H19 allele-specific expression was not altered in this case (see Table 2). Our data, specifically those from cases #5 and 6, informative for both IGF2 and H19, thus suggest independent transcriptional regulation of these two genes in breast tissue, in contrast to a proposed regionally controlled regulation of IGF2 and H19 expression [13]. Although this 'enhancer competition model' is supported by the observations of several studies in mice ([9] for review), also less compatible data are emerging suggesting, like our study, that the allele-specific transcriptional regulation of these two genes is not as closely linked as previously assumed [22,28,29].

Another remarkable example of the versatility in the regulation of IGF2 expression was displayed in one of the in vitro tumor tissue fibroblast samples (case #4). While the fibroblast culture revealed monoallelic expression of the B-allele, the corresponding in vivo tumor sample showed expression of only the A-allele. Subsequent allelotypic analysis of fibroblast DNA showed that this was due to a complete loss of the non-expressed A-allele in this culture (not shown).

In summary, we have presented data showing that the allelic expression pattern of IGF2 and H19 is regulated in a similar fashion in malignant and adjacent histologically normal breast tissue. Our data furthermore indicate that IGF2, but not H19, is prone to loss of imprinting in breast cancer. Extended studies are required, however, to further substantiate these findings.

Acknowledgements: We like to thank Dr. B. Tycko (Department of Pathology, Divisions of Oncology and Neuropathology, Columbia University, New York, USA), Dr. T. Moulton (Department of Pediatrics, Division of Pediatric Hematology-Oncology, Columbia University, New York, USA) and Dr. A.J.M.H. Verkerk (Lab. for Exp. Patho-Oncology, Daniel den Hoed Cancer Center) for their information concerning the H19 polymorphic *Avi*II location and sequence. This study was supported by the Dutch Cancer Society Grants DDHK 91-15 and DDHK 94-867.

References

[1] Bièche, I. and Lidereau, R. (1995) Genes Chromosomes Cancer 14, 227–251.
[2] Garin-Chesa, P., Old, L.J. and Rettig, W.J. (1990) Proc. Natl. Acad. Sci. USA 87, 7235–7239.
[3] Kaczmarek, J., Castellani, P., Nicolo, G., Spina, B., Allemanni, G. and Zardi, L. (1994) Int. J. Cancer 58, 11–16.
[4] Cullen, K.J., Smith, H.S., Hill, S., Rosen, N. and Lippman, M.E. (1991) Cancer Res. 51, 4978–4985.
[5] van Roozendaal, C.E.P., Klijn, J.G.M., van Ooijen, B., Claassen, C., Eggermont, A.M.M., Henzen-Logmans, S.C. and Foekens, J.A. (1996) Int. J. Cancer 65, 120–125.
[6] Macaulay, V.M. (1992) Br. J. Cancer 65, 311–320.
[7] Ellis, M.J.C., Singer, C., Hornby, A., Rasmussen, A. and Cullen, K.J. (1994) Breast Cancer Res. Treat. 31, 249–261.
[8] Franklin, G.C., Adam, G.I.R. and Ohlsson, R. (1996) Placenta 17, 3–14.
[9] Looijenga, L.H.J., Verkerk, A.J.M.H., de Groot, N., Hochberg,

A.A. and Oosterhuis, J.W. (1997) Mol. Reprod. Dev. 46, 419–439.
[10] Zemel, S., Bartolomei, M.S. and Tilghman, S.M. (1992) Nat. Genet. 2, 61–65.
[11] Giannoukakis, N., Deal, C., Paquette, J., Goodyer, C.G. and Polychronakos, C. (1993) Nat. Genet. 4, 98–101.
[12] Zhang, Y. and Tycko, B. (1992) Nat. Genet. 1, 40–44.
[13] Bartolomei, M.S., Webber, A.L., Brunkow, M.E. and Tilghman, S.M. (1993) Genes Dev. 7, 1663–1673.
[14] Latham, K.E., McGrath, J. and Solter, D. (1995) Int. Rev. Cytol. 160, 53–98.
[15] Brenton, J.D., Viville, S. and Surani, M.A. (1995) Cancer Surveys 25, 161–171.
[16] Weksberg, R., Shen, D.R., Fei, Y.L., Song, Q.L. and Squire, J. (1993) Nat. Genet. 5, 143–150.
[17] Jarrard, D.F., Bussemakers, M.J.G., Bova, G.S. and Isaacs, W.B. (1996) Clin. Cancer Res. 1, 1471–1478.
[18] Verkerk, A.J.M.H., Ariel, I., Dekker, M.C., Schneider, T., van Gurp, R.J.H.L.M., Gillis, A.J.M., Oosterhuis, J.W., Hochberg, A.A. and Looijenga, L.H.J. (1997) Oncogene 14, 95–107.
[19] Dugimont, T., Curgy, J.-J., Wernert, N., Delobelle, A., Raes, M.-B., Joubel, A., Stehelin, D. and Coll, J. (1995) Biol. Cell 85, 117–124.
[20] Eversole-Cire, P., Ferguson-Smith, A.C., Surani, M.A. and Jones, P.A. (1995) Cell Growth Differ. 6, 337–345.
[21] Yballe, C.M., Vu, T.H. and Hoffman, A.R. (1996) J. Clin. Endocrinol. Metab. 81, 1607–1612.
[22] Douc-Rasy, S., Barrois, M., Fogel, S., Ahomadegbe, J.C., Stehelin, D., Coll, J. and Riou, G. (1996) Oncogene 12, 423–430.
[23] Zhang, Y., Shields, T., Crenshaw, T., Hao, Y., Moulton, T. and Tycko, B. (1993) Am. J. Hum. Genet. 53, 113–124.
[24] McCann, A.H., Miller, N., O'Meara, A., Pedersen, I., Keogh, K., Gorey, T. and Dervan, P.A. (1996) Hum. Mol. Genet. 5, 1123–1127.
[25] Hu, J.-F., Vu, T.H. and Hoffman, A.R. (1998) J. Biol. Chem. 272, 20715–20720.
[26] Wu, H.-K., Squire, J.A., Catzavelos, C.G. and Weksberg, R. (1997) Biochem. Biophys. Res. Commun. 235, 123–129.
[27] Okamoto, K., Morison, I.M., Taniguchi, T. and Reeve, A.E. (1997) Proc. Natl. Acad. Sci. USA 94, 5367–5371.
[28] Rainier, S., Dobry, C.J. and Feinberg, A.P. (1995) Cancer Res. 55, 1836–1838.
[29] Uyeno, S., Aoki, Y., Nata, M., Sagisaka, K., Kayama, T., Yoshimoto, T. and Ono, T. (1996) Cancer Res. 56, 5356–5359.