# Correlation between chromosome abnormalities and genomic imprinting in developing human

## 1) Frequent bialleleic expression of insulin-like growth factor II (IGF2) in gynogenetic Ovarian Teratomas: Uncoupling of H19 and IGF2 imprinting

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Human uniparental gestations such as gynogenetic ovarian teratomas provide a model to evaluate the integrity of parent-specific gene expression - i.e. imprinting - in the absence of a complementary parental genetic contribution. The few imprinted genes characterized so far include the insulin-like growth factor-2 gene (IGF2) coding for a fetal growth factor and H19 gene whose normal function is unknown but it is likely to act as an mRNA. IGF2 is expressed by the paternal allele and H19 by the maternal allele. This reciprocal expression is quite interesting because both H19 and IGF2 genes are located close to each other on chromosome 11p15.5. *In situ* RNA hybridization analysis has shown variable expression of the H19 and IGF2 alleles according to the tissue origin in 11 teratomas. Especially, Skin, derivative of ectoderm, is expressed conspicuously. We examined imprinting of H19 and IGF2 in teratomas using PCR and RT-PCR of exonic polymorphism. H19 and IGF2 transcript could be expressed either biallelically or monoallelically in the teratomas. Biallelic expression (i.e., loss of imprinting) of IGF2 occurred in 5 out of 6 mature teratomas and 1 out of 1 immature teratoma. Biallelic expression of H19 occurred in 4 out of 10 mature teratomas and 1 out of 1 immature teratoma. Expression levels of H19 and IGF2 transcript using the semi-quantitative RT-PCR had no relation between monoallelic and biallelic expression. Moreover, IGF2 biallelic expression did not affect allele-specificity or levels of H19 expression. These results demonstrate that both genes, H19 and IGF2, can be imprinted, expressed and regulated independently and individually of each other in ovarian teratoma.

**Keywords:** Genomic imprinting, H19 gene, IGF2 gene, Loss of Imprinting, Ovarian teratoma

## INTRODUCTION

Genomic imprinting implies the existence of a process that is fully reversible in which there is a gamete-specific modification in the parental genome that leads to observable functional differences between the paternal and maternal genomes in the somatic cells of the offspring (Hall, 1990; Sapienza, 1995; Barlow, 1995; John and Surani, 1996; Hoffman *et al.*, 1996). An imbalance of parental chromosomes in the embryo, or aberrant imprinting of these genes is implicated in an increasing number of genetic disorders (Reik and Surani, 1989; Nicholls, 1989) and may be involved in carcinogenesis (Wilkins, 1988; Feinberg, 1993; Hoffman, 1996; Glassman *et al.*, 1996). Among imprinting genes, IGF2 and H19 (Rainier *et al.*, 1993: Zhang *et al.*, 1993: Sasaki *et al.*, 1995), tightly linked on human chromosome 11, are of special interest because of their reciprocal imprinting. The IGF2 is expressed from the paternally derived allele, whereas the H19 is expressed from the maternally derived allele (Rainier *et al* 1993: Ogawa *et al.*, 1993: Zhang *et al.*, 1993). These genes are related to the differentiation of fetal tissues, embryonal development and tumorigenesis (Goshen *et al.*, 1993; Lustig *et al.*, 1994; Sasaki *et al.*, 1995; Kondo *et al.*, 1995; Wada *et al.*, 1995; Hibi *et al.*, 1996). IGF2, a potent growth factor and mitogen is produced locally, maintaining and enhancing tumor growth (Thompson *et al.*, 1990: Albrecht *et al.*, 1996: Yballe *et al.*, 1996). H19 may act as an RNA since no protein product could be evidenced (Brannan *et al.*, 1990). Although the precise function of this gene is unknown, H19 RNA has been shown to suppress tumor morphology in transfected tumor cells (Hao *et al.*, 1993), which is highly expressed during fetal development

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(Goshen *et al.*, 1993; Lustig *et al.*, 1994).

Tissues of uniparental gestations provide a powerful tool to define the extent of importance delineated by a single parental genome. There are no natural examples of genuine gynogenetic conceptions, although ovarian teratoma have a 46, XX chromosome complement, all of which is maternally derived. This suggests that these tumors arise by abnormal development following the first and second meiotic division (Deka *et al.*, 1990). These tumors consist of differentiated ectodermal, mesodermal and endodermal structures but have no placental elements (Surti *et al.*, 1990). Loss of imprinting (LOI) of H19 and IGF2, resulting in biallelic expression, has been reported in a patient of several tumor (Ogawa *et al.*, 1993; Rainier *et al.*, 1993; Steenman *et al.*, 1994; Taniguchi *et al.*, 1995) and has been suggested to play a role in tumor progression. The occurrence of LOI of genes would theoretically double the gene dosage with a possibility of doubling the transcription rate. More recently, it has been shown that, in Wilms' tumor, loss of imprinting (LOI) of IGF2 is associated with reduction of H19 mRNA (Steenman *et al.*, 1994; Taniguchi *et al.*, 1995). We, therefore, examined the imprinting status of H19 and IGF2 and the relationship between the expression of the IGF2 and H19 locus in 10 mature teratomas and one immature teratoma. Our results have shown that the expression of IGF2 mRNA was co-localized with the expression of H19 mRNA and low level expression was seen in ovarian teratomas. IGF2 was activated biallelically in most mature teratomas and immature teratoma at the IGF2 locus. H19 was expressed biallelically in 4 out of 10 heterozygotic mature teratoma and one immature teratoma at the H19 locus. However, there is no difference between biallelic or monoallelic expressions in the expression levels of both H19 as well as IGF2 genes in teratomas. It seems that the imprinting and the expression of both genes can be occurred independently of each other in ovarian teratoma.

## MATERIALS AND METHODS

### Tissue samples

Tissues diagnosed during or after surgery were obtained as fresh specimens and were investigated histologically and morphologically at the pathology department in Dongsan hospital. The total number of specimens of each type studied were one immature and ten mature teratomas.

### Tissue preparation

Tissues were fixed in buffered 4% paraformaldehyde for 20 h, washed in PBS (-), and paraffin embedded for teratomas.

### Preparation of riboprobes

H19 and IGF2 riboprobes were made by RT-PCR, cloning, and *in vitro* transcription. PCR primers, sizes of fragments, and amplification conditions were previously reported:

H19 primer (Zhang *et al.*, 1993)
sense: 5'-GAAAAAGCCCGGGCTAGGAC-3'
antisense: 5'-GCGTCACCAAGTCCACTGTG-3'

IGF2 primer (Davies, 1993)
sense: 5'-TCCTGGAGACGTACTGTGCTA-3'
antisense: 5'-GGTCGTGCCAATTACATTTCA-3'.

PCR products were cloned into a pGEM-T vector and transcripted from the linearlized template using SP6 and T7 RNA polymerase labelling with digoxigenin-UTP to H19 and IGF2 by using RNA labelling mixture (Boeringer Mannheim).

### *In situ* hybridization

Serial 5 μm sections from paraffin-embedded mature teratomas and immature teratoma were mounted on 2% amin-propyl triethoxysilane (Sigma) in acetone-coated slides. Paraffin sections were pretreated at 60°C for 3 h, deparaffinized, and treated by 0.2 N HCl, then by 15 μg/μl proteinase K (sigma). After rinsing with 0.1% DEPC treated water, sections were prehybridized for 3 h at 50°C with prehybridization solution (4 × SSC, 10% Dextran sulfate, 1 × Denhardt's solution, 2 mM EDTA, 50% formamide, 500 μg/μl denaturated ssDNA). Hybridizations with labelled riboprobes were performed for overnight at 50°C. The sections were washed stringently and detected. For detection the digoxigenin labelled H19 and IGF2 probes hybridized with H19 and IGF2 transcripts in sections, anti-dig-AP diluted 1:500 in 4 × SSC/1% BSA was treated for 1 h at room temperature. After the color reaction by NBT/BCIP, the signals were observed under light microscope (Nikon). 0.5% methyl green was used as a counter stain. The controls were maintained with sense riboprobes and without riboprobes, only negligible signals were recorded (Data not shown).

### Nucleic acid preparation

Genomic DNA was prepared from ovarian teratoma by treatment with proteinase K (final concentration, 100 μg/μl) and 0.5% SDS, followed by repeated phenol/chloroform extraction. Total cellular RNA was prepared as RNA Zol B (TEL-Test. INC). To generate cDNA, 2 μg total RNA was reverse-transcribed in 20 μl reaction mixture containing 5 mM $MgCl_2$, 1 mM dNTP, 1 U/μl RNase inhibitor, 2.5 U/μl MuLV reverse transcriptase, and 2.5 μM oligo d $(T)_{16}$ for 1

h at 42°C.

### Determination of allelic specific gene expression of H19 and IGF2

A set of primers flanking the 3' last exon of H19 were designated to determine allele-specific expression:

H19 primer (Zhang and Tycko, 1993)
S3 (sense): 5'-TACAACCACTGCACTACCCTG -3'
AS3 (antisense): 5'-TGGAATGCTTGAAGGCTGCT-3'.

The *ApaI* RFLP was analysed by using primers flanking the exon 9 of IGF2:

IGF2 primer (Ogawa *et al.*, 1993)
P2 (sense): 5'-CTTGGACTTTGAGTCAAATTGG-3'
P3 (antisense): 5'-GGTCGTGCCAATTACATTTCA-3'.

PCR was performed using *Taq* polymerase (Promega) in 40 μl PCR buffer containing each of the primers at a final concentration of 0.5 μM, with an initial denaturation of 5 min at 94°C followed by 35 cycles of 1.5 min at 94°C, 2 min at 58°C, 2 min at 72°C, and an additional extension of 72°C for 7 min for H19, and an initial denaturation of 5 min at 95°C followed by 35 cycles of 1.5 min at 94°C, 2 min at 55°C, 2 min at 72°C, and additional extension of 72°C for 7 min for IGF2. The PCR products were purified by using wizard PCR preps DNA purification system (Promega), digested with 30 U of *RsaI* for H19 and with *ApaI* for IGF2 at 37°C for overnight and analysed by electrophoresis using 2% agarose gel and subsequent ethidium bromide staining.

### Detection of H19 and IGF2 mRNA expression using RT-PCR

cDNA synthesis was performed with 2-4 μg of total RNA in a reaction volume of 40 μl and incubated for 1 h at 42°C. PCR reactions were performed with 4 μl of the cDNA reaction mixture. The cDNA amplified with the PCR using the oligonucleotide primer pairs had the following sequences:

H19 (Jinno *et al.*, 1995)
sense: 5'-GGCTCCCAGAACCCACAAC A-3'
antisense: 5'-TGATGATGAGTCCAGGGCTC-3'

IGF2 (Giannoukakis *et al.*, 1993)
sense: 5'-CTTGGACTTTGAGTCAAATTGG-3'
antisense: 5'- CCTCCTTTGGTCTTACTGGG-3'

GAPDH (Lucibello *et al.*, 1993)
sense: 5'-CGTCTTCACCACCATGGAGA-3'
antisense: 5'-CGGCCATCACGCCACAGTTT-3'.

GAPDH served as an internal control of the reaction. PCR amplification was carried out under the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C for H19 (35 cycles), 1 min at 92°C, 1 min at 55°C, 2 min at 72°C for IGF2 (30 cycles), and 1 min at 94°C, 1 min at 60°C, 1 min at 72°C for GAPDH (30 cycles). A 10 μl sample of the PCR reaction mixture was separated on a 1.2% agarose gel containing 0.1 μg/ml ethidium bromide and viewed by transillumination with UV light and photographed. Quantification was performed by using the density measuring function of the Optimas version 5.2 image analyser system (Optimas).

## RESULTS AND DISCUSSION

### H19 and IGF2 expression in ovarian teratoma

Cellular localization and expression level of H19 and IGF2 transcripts was analyzed by *in situ* RNA hybridization using paraffin-embedded tissues. The IGF2 gene is maternally imprinted in humans, with only the paternal allele being expressed in tissues, so it has to be silent in ovarian teratomas, containing exclusively maternally chromosomes. However, our data show that mature teratomas, as well as one immature teratoma with areas of immature cartilage, have shown variable levels of expression on *in situ* RNA hybridization with IGF2 transcripts (Fig. 1). This result is in disagreement with previous study demonstrating teratomas had no specific signal on *in situ* hybridization with H19 and IGF2 (Mutter *et al.*, 1993). Our results demonstrate that functional imprinting of IGF2 gene seems to represent another loss of imprinting in teratoma.

Representative tissues in these *in situ* RNA hybridization studies included derivatives of all three embryonic layers: (1) ectoderm - i.e., skin and neural tissue ; (2) mesoderm - i.e., cartilage and smooth muscle ; (3) endoderm - i.e., respiratory epithelium. Our findings by *in situ* RNA hybridization have shown that expressions of IGF2 in teratomas were almost weak in endodermal, mesodermal, and ectodermal derivatives, with the exception of epidermis cells in skin, which is of ectodermal origin (Fig. 1). Lack of IGF2 expression in gynogenetic teratomas cannot be ascribed to lack of tissues in which this RNA is usually present, as one mature teratoma examined by *in situ* RNA hybridization (data not shown) contains choroid plexus, a tissue that usually transcribes IGF2 from both parental alleles (DeChiara *et al.*, 1991), which highly express IGF2. Moreover, cellular localization

was almost paralleled in H19 and IGF2 transcripts (Fig. 1). These results have shown that expressions of H19 and IGF2 have tissue-specificity and the same cells and same maternal chromosomes coexpressed both H19 and IGF2 transcripts in ovarian teratomas.

## Biallelic expression (i.e. loss of imprinting) of H19 and IGF2

In order to gain both a more detailed understanding as to the existence of imprinting in mammals and further insights into the mechanisms regulating the expression of imprinted genes, we analyzed the allele-specific expression of IGF2 and H19 in ovarian teratomas. H19 was shown to be imprinted with maternal and IGF2 with paternal monoallelic expressions in normal individuals. Ovarian teratomas have been demonstrated to have two maternal sets of haploid chromosomes - i.e., gynogenetic. Regardless of these results, this data like results of *in situ* RNA hybridization presents another phenomenon the genomic imprinting status of H19 and IGF2 in teratomas.

Genomic DNA and cDNA from the ten mature teratomas and one immature teratoma, was subjected to amplification by PCR using primers flanking an *RsaI* polymorphic sites in exon 5 of the H19. To eliminate the possible amplification of contaminating genomic DNA during RT-PCR, the cDNA was amplified using primers that spanned the last intron of the H19. In mature teratoma examined, four of ten cases with heterozygosity revealed biallelic H19 expression (Fig. 2, Table 1). Immature teratoma exhibited biallelic expression (Fig. 2, Table 1). Similarly, we used a PCR amplification to assess which alleles were present at a polymorphic *ApaI* site in exon 9 of the IGF2. Five cases of six mature teratomas with heterozygosity, exhibited biallelic IGF2 expression (Fig. 3, Table 1). Immature teratoma was also shown biallelic expression (Fig. 3, Table 1). Our findings indicated that IGF2 is expressed biallelically in the most gynogenetic

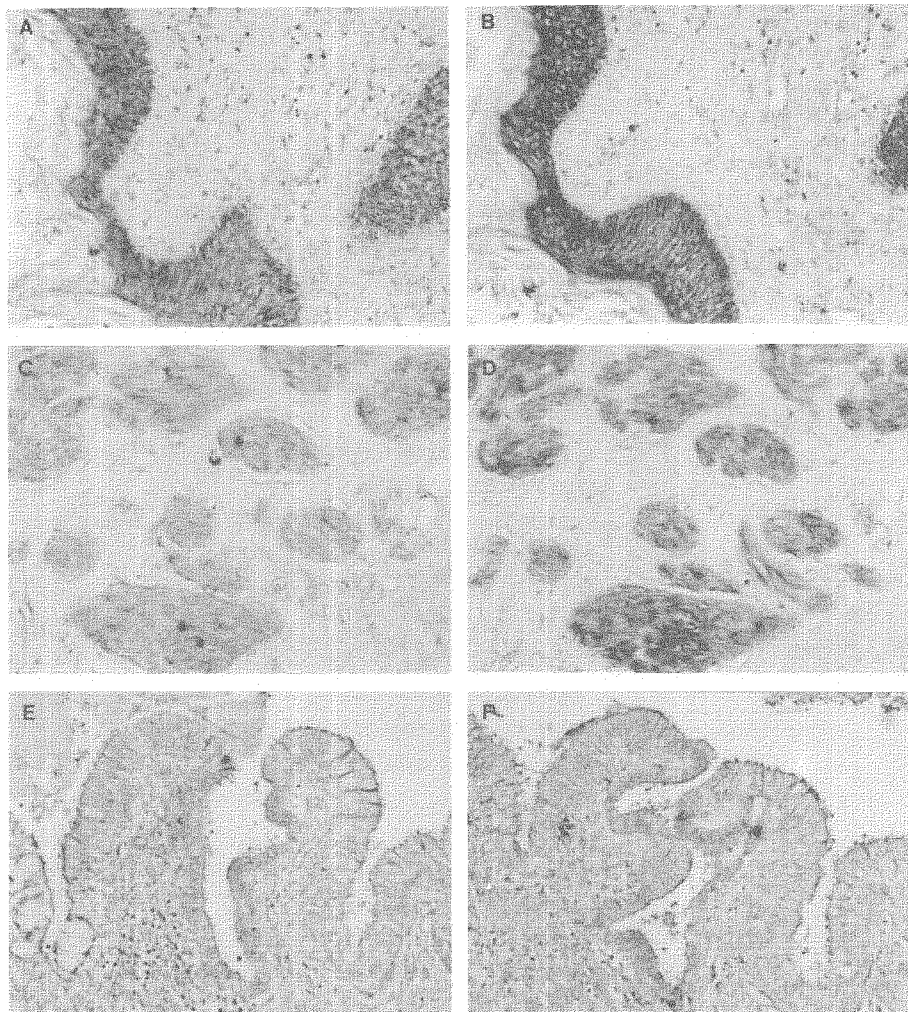


**Fig. 1.** *In situ* hybridization analysis of H19 (A, C and E) and IGF2 (B, D and F) RNA probe localization in ovarian teratoma. A, B, epidermis cells in skin, which is of ectodermal origin, expressed prominently. C, D, smooth muscle, which of mesodermal origin and E, F, respiratory epithelium, which is of endodermal origin, expressed weakly. Cellular localization was almost paralleled in H19 and IGF2 transcript. Magnification 50X.

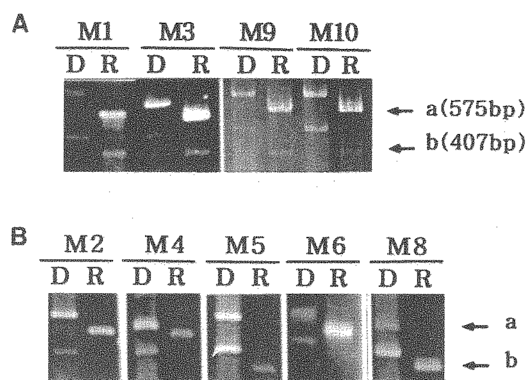


**Fig. 2.** Representative examples of H19 expression in teratomas using the *RsaI* polymorphism. For each sample, the left lane (D) contains the PCR product obtained with gDNA while the right lane (R) shows the cDNA PCR product. A, H19 biallelic expression in mature teratomas and B, H19 monoallelic expression in mature teratomas. The gDNA alleles are larger than the corresponding cDNA alleles because the primers bracket a small intron.

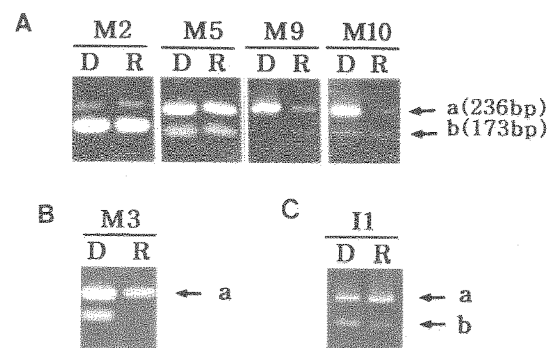


**Fig. 3.** Representative examples of IGF2 expression in teratomas using the *ApaI* polymorphism in exon 9 of the gene. For each sample, the left lane (D) contains the PCR product obtained with gDNA while the right lane (R) shows the cDNA PCR product, both after digestion with *ApaI*. A, IGF2 biallelic expression inmature teratomas, B, IGF2 monoallelic expression in mature teratomas and C, IGF2 biallelic expression in immature teratoma.

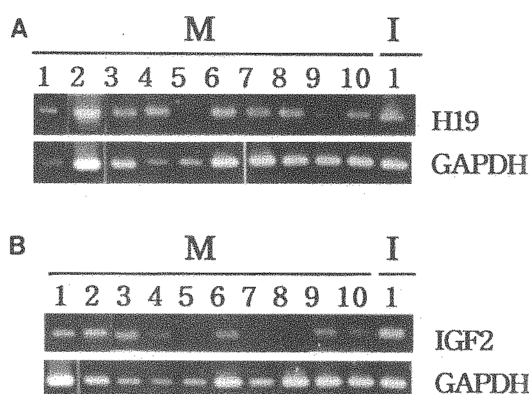


**Fig. 4.** Semi-quantitative RT-PCR analysis of H19/GAPDH and IGF2/GAPDH mRNA in ovarian teratomas. mRNAs were transcribed into cDNA and the products amplified by PCR using conditions that allow a quantitative evaluation of the data (see Materials and Methods for details). Average relative intensity of each band was scored using a Optimas version 5.2 image analyser system.

**Table 1. Expression of *H19* and *IGF2* in 11 ovarian teratomas.**

| Teratoma | *H19/RsaI* | | *IGF2/ApaI* | | Expression | Expression |
|---|---|---|---|---|---|---|
| | DNA | RNA | DNA | RNA | H19/GAPDH | IGF2/GAPDH |
| [a]MT1 | [c]a/b | [d]a/b | a/a | a/- | 0.9 | 0.39 |
| MT2 | a/b | a/- | a/b | a/b | 0.81 | 0.35 |
| MT3 | a/b | a/b | a/b | a/- | 0.81 | 1.38 |
| MT4 | a/b | a/- | a/a | a/- | 3.07 | 1.02 |
| MT5 | a/b | -/b | a/b | a/b | 0.40 | 0.15 |
| MT6 | a/b | a/- | a/b | a/b | 0.94 | 0.32 |
| MT7 | a/b | -/b | a/a | a/- | 0.47 | 0.13 |
| MT8 | a/b | -/b | a/a | a/- | 0.75 | 0.24 |
| MT9 | a/b | a/b | a/b | a/b | 0.39 | 0.44 |
| MT10 | a/b | a/b | a/b | a/b | 0.79 | 0.29 |
| [b]IT1 | a/b | a/b | a/b | a/b | 1.21 | 1.00 |

[a] MT; Mature teratoma. [b] IT; Immature teratoma.
[c] a/b, heterozygote; a/a or b/b, homozygote.
[d] a/-, expression of a allele; -/b, expression of b allele; a/b, expression of both alleles.

teratomas, and it can be speculated as the results of loss of IGF2 imprinting. Wutz *et al.* (1997) have shown that differentially methylated regions in the Igf2r gene can carry a critical imprinting signal. According to this report, there is region 1 and 2 that are sites of differential methylation, loss of imprinting is appeared by deletion of region 2 that abolished the expression of the antisense message, hence paternal or maternal repression.

Our data show that the frequency of biallelic expression, i.e. loss of imprinting, in IGF2 and H19 was 86% and 45%, respectively (Table 1). Loss of imprinting (LOI) has been suggested to play a role in tumor progression. Among certain embryonal tumors of childhood, LOI is not a frequent event (Davies, 1993; Li *et al.*, 1995). However, LOI of H19 and IGF2 occurs frequently in Wilms' tumors (Ogawa *et al.*, 1993; Rainier *et al.*, 1993), Ewing's sarcomas (Zhan *et al.*, 1995), and lung carcinomas (Kondo *et al.*, 1995). Our data have indicated that ovarian teratomas with only maternal allele show IGF2 expression and IGF2 mostly appeared as a biallelic expression.

### Expression levels of H19 and IGF2 transcripts appear independently regulated

Total cellular RNA was isolated from ten mature teratomas and one immature teratoma and examined by semi-quantitative RT-PCR to know the difference between biallelic and monoallelic expression in H19 and IGF2 transcripts. The levels of H19 and IGF2 transcripts were determined by checking the density of each line using density measuring function of the Optimas version 5.2 image analyser system (Optimas corp., USA). The relative intensity of H19 and IGF2 was obtained by a H19/GAPDH or IGF2/GAPDH ratio (Table 1). A representative expression pattern is presented in Fig. 4. The H19 transcript was observed in ten cases of mature teratomas and in one case of immature teratoma. When detectable, the wide range of expression of H19 transcript was detected among each cases. A loss of imprinting would be theoretically increased 2-fold the gene dosage. However, our data have shown that biallelic expression of H19 transcript was approximately similar to the monoallelic expression (Table 1). The IGF2 transcript was also observed in ten cases of mature teratomas and one case of immature teratoma. IGF2 transcript were less abundant than H19 transcripts but there were also variations in their levels in teratomas. Biallelic expression is not necessarily associated with the highest levels of IGF2 transcripts (Table 1). This is similar to those by Voutilainen *et al.* (1994) in which IGF2 and H19 levels were found to be regulated in parallel in cultured adrenal cells.

Our data have shown one case with a low IGF2 transcript level, had a low H19 transcript level indicating no inverse correlation between H19 and IGF2 expressions. In the study of Ewing sarcoma, Zhan *et al.* (1995) also found no correlation between loss of imprinting of H19 and IGF2 transcript levels. These data are different from those reported in Wilms' tumor (Steenman *et al.*, 1994; Taniguchi *et al.*, 1995); biallelic expression of IGF2 was linked to a striking reduction in H19 mRNA level.

The imprinting of H19 and IGF2 is a precisely localized phenomenon. There are two possible models to explain this type of "circumscribed" imprinting. First, each imprinted gene may contain its own individual imprinting site. Second, a local imprinting control element may control the imprinting of a cluster of genes (John and Surani, 1996; Reik and Constancia, 1997). The expression patterns of H19 and IGF2 in most normal tissues and Wilms' tumor have been taken the second model with the imprint possibly mediated through a competitive enhancer/promoter system (Steenman *et al.*, 1994; Taniguchi *et al.*, 1995; Leighton *et al.*, 1995). In our study, one of the mature teratomas expressed H19 monoallelically and IGF2 biallelically and vice versa. Two of the mature teratomas had monoallelic expression of H19 but IGF2 expression could not be nearly detectable. Thus, in the ovarian teratomas, establishment of H19 and IGF2 functional imprinting and expression seems to occur as individual and independent events, at least allele-specific expression of IGF2 appears not to be affected by H19 expression.

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