The expression of the imprinted H19 and IGF-2 genes in human bladder carcinoma

Michael Elkin[a], Alexander Shevelev[b], Ekkehard Schulze[b], Mark Tykocinsky[b], Mark Cooper[c], Ilana Ariel[d], Dov Pode[e], Eliezer Kopf[f], Nathan de Groot[a], A. Hochberg[a,*]

[a]*Department of Biological Chemistry, The Silberman Institute of Life Sciences, The Hebrew University, Jerusalem 91904, Israel*
[b]*Institute of Pathology, Case Western Reserve University, Cleveland, OH, USA*
[c]*Department of Medicine, Case Western Reserve University, Cleveland, OH, USA*
[d]*Department of Pathology, Hadassah University Hospital, Mount Scopus, Jerusalem, Israel*
[e]*Department of Urology, Hadassah University Hospital, Ein-Karem, Jerusalem, Israel*
[f]*Department of Biochemistry, Weizmann Institute of Science, Rehovot, Israel*

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Abstract The imprinted H19 gene is highly expressed in human embryos, fetal tissues and is nearly completely shut off in adults. However, it is reexpressed in a number of tumors including bladder carcinoma, demonstrating that H19 RNA is an oncofetal RNA. Tumors induced by injection of bladder carcinoma cell lines express H19 in contrast to the cells before injection. These observations support the notion of a positive correlation between H19 expression and bladder carcinoma. Loss of imprinting of H19 and IGF-2 was observed in samples of human bladder carcinoma.

Key words: Genomic imprinting; Bladder carcinoma; H19; IGF-2; Mono-allelic expression; Bi-allelic expression

1. Introduction

The human H19 gene is a paternally imprinted, maternally expressed, gene. It is transcribed by RNA polymerase II, its transcripts are processed and transported to the cytoplasm but are not associated with ribosomes and not translated [1]. The function of the H19 RNA is unknown. However, non-translated RNAs may act as riboregulators. These include the H19 RNA, the XIST gene product [2], IPW gene product [3] the enod 40 RNA [4] and RNA sequences beyond the termination codon of muscle specific mRNAs as in tropomyosin mRNA [5].

The relative few genes known to be imprinted tend to appear in clusters in certain chromosomal regions. The H19 gene in the human is located on the chromosome 11p15.5 in close physical proximity to the reciprocally imprinted IGF-2 gene [6]. IGF-2 is a potent mitogene and is highly expressed in several tumors [7]. Several imprinted genes are clustered on human chromosome 15 in an area critical for the Prader-Willi and Angelman syndromes [8].

The human H19 gene is highly expressed during pregnancy in the extraembryonic tissues (placenta) in the embryo proper and in several fetal tissues, including the bladder [9]. Its expression is nearly completely shut off postnatally. A noteworthy exception is the uteral endometrium of the adult female which expresses H19, the highest expression is during the secretory phase of the menstrual cycle [10].

The H19 gene is reactivated during the development of some tumors which express the H19 gene during embryogenesis and fetal life. H19 was found to be expressed in embryonic tumors like Wilm's tumor, rhabdomyosarcoma and hepatoblastoma [11] in trophoblastic tumors [12], testicular germ cell tumors [13], lung tumors [14] and in bladder carcinoma [15]. In all these tumors, except bladder carcinoma, loss of imprinting was reported. In analogy to oncofetal proteins such as α-feto-protein and carcino embryonic antigen (CEA) the H19 gene product can be considered an oncofetal RNA [16]. Actually the H19 gene in the mouse was discovered as a gene under coordinate regulation with the α-feto-protein in the liver [17]. Very little is known about a possible functional relationship between H19 expression and tumorigenesis. It has been suggested that H19 acts as a tumor suppressor gene but the above-mentioned findings do not support this proposal.

The purpose of the experiments described in this paper is to provide new data concerning the relationship between H19 expression and the loss of imprinting of H19 and IGF-2 in bladder cancer.

2. Materials and methods

2.1. Cell culture

Bladder carcinoma cell lines HT-1376; UM-UC-3 and T24P were obtained from the American Type Culture Collection (ATCC), EJ28 cell line was obtained from the German Cancer Research Centre, Heidelberg, Germany. C-2, a myoblast mouse cell line was kindly provided by Prof. David Jaffe, Dept. of Cell Biology, Weizmann Institute of Science, Rehovot, Israel.

The medium used was DMEM.F12 (1:1) containing 10% fetal calf serum (Beit Haemek, Israel) 25 mM HEPES pH 7.4; penicillin (180 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.2 μg/ml).

All cultures were maintained in polystyrene culture dishes (NUNC) and were transferred every 4 days by treatment with 0.05% trypsin-EDTA solution (Beit Haemek, Israel).

2.2. Animals

12 female CD-1 athymic nude (*Nu/Nu*) mice were used for the present study. At 50 days of age they were divided into 4 groups of 3 mice each.

2.3. Experimental design

Cells from the cell lines UM-UC-3; T24P; HT-1376 and EJ28 were suspended in culture medium without serum (5×10^6 cells/ml) and injected into mice of groups 1 to 4, respectively. Each nude mouse received two bilaterally subcutaneous injections in the dorsal flank region (1×10^6 cells/site). The tumors formed were isolated after they reached

*Corresponding author. Fax: (972) (2) 61-0250.

Abbreviations: IGF-2, insulin-like growth factor II gene; IPW, imprinted gene in the Prader-Willi syndrome region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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the size of 1 cm diameter. Portions of the tumors were either preserved at −80°C for RNA extractions or fixed in formalin for histological examination and in situ hybridization, or kept in saline until isolation of tumor cells. Tumor cells were recovered as in [18] and maintained as described above.

2.4. In situ hybridization

The preparation of H19 antisense riboprobe and the in situ hybridization were performed as previously described by us [12].

2.5. Isolation of RNA and DNA

Total cellular RNA was isolated from tissue by the guanidinium-thiocyanate procedure and from the cells by guanidium phenol chloroform method [19]. DNA was isolated as described in [20].

2.6. RT-PCR analysis

For synthesis of cDNAs, random hexamer primers (Boehringer, Mannheim, Germany) were used to initiate reverse transcription of 0.2 μg total RNA with 50 units M-MLV Reverse Transcriptase (Gibco BRL). PCR amplification reactions for the genes investigated were performed in a Mini-Cycler (MJ Research, Watertown, MA, USA) in 50 μl volume using Taq DNA polymerase and buffer (Boehringer, Mannheim, Germany).

The primer sequences, number of cycles and conditions for amplifications of cDNA of the H19 gene were as described in [21]. The amplification of GAPDH cDNA was performed using GAPDH primers (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, for 24 cycles.

35 cycles of amplification of IGF-2 cDNA were carried out as described in [22].

Oligonucleotide primer sequence for PCR of Ha-ras-1 were TGACGGAATATAAGCTGGTG (upstream) and GTACTGGTGGATGTCCTCAA (downstream). The PCR conditions were an initial denaturation of 3 min and subsequent denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension for 1 min at 75°C (30 cycles) followed by 5 min final extensions at 75°C.

Aliquots of 10 μl of the amplification products were separated on 2% agarose gels and visualized by ethidium bromide staining.

Only those samples of RNA that gave completely negative results in PCR without reverse transcriptase were further analyzed. In addition, amplification of H19 from DNA produced a larger product than amplification from mRNA because of the presence of 2 introns in the comparable DNA sequence.

2.7. Analysis of tissues for mono- or bi-allelic expression of H19 and IGF-2

Tumor tissues were collected from patients with bladder carcinoma.

DNA and reverse transcribed total RNA from each tissue was analysed by RFLP assay using the polymerase chain reaction and *Rsa*I polymorphism in the H19 gene, as described in [21] and an *Apa*I polymorphism in IGF-2 as described in [22].

3. Results

3.1. Mono- and bi-allelic expression of H19 and IGF-2 in human bladder carcinoma

As it has been shown recently a high level of H19 transcripts can be detected in the fetal urinary bladder [15]. Normal adult urothelium and low grade, non-invasive transitional cell bladder carcinomas do not express H19, but in grade II and III bladder carcinomas, invasive transitional cell carcinomas, and in situ bladder carcinomas, reactivation of H19 expression was demonstrated. H19 was proposed as a tumor marker for the progression to more malignant stages in bladder cancer [15].

As loss of imprinting is frequently observed in embryonal tumors [23], the allelic mode of the expression of H19 and IGF-2 was investigated.

Out of 12 bladder carcinoma biopsies, 4 were informative as they showed *Rsa*I restriction polymorphism in the H19 gene. Two showed *Apa*I restriction polymorphism in the IGF-2 gene

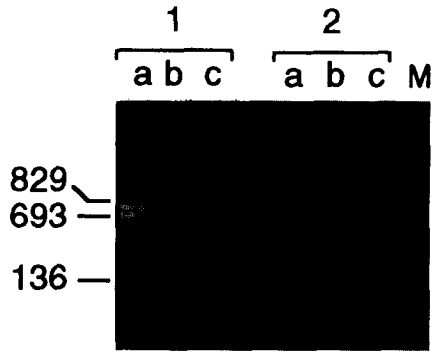

Fig. 1. Allelic mode of H19 expression in two out of four cases of bladder carcinoma, showing poymorphism in the H19 gene: lanes a = genomic DNA PCR products restricted with *Rsa*I; lanes b = RT-PCR products before and lanes c = after restriction with *Rsa*I, demonstrating monoallelic expression in tumor 1 and bi-allelic expression in tumor 2. Lane M = molecular weight marker VI (Boehringer-Mannheim). The size of the DNA (bp) is indicated.

and it is of interest to note that these two cases belonged to the group of four which were heterozygous for the H19 gene. In two of the four H19 informative tumors bi-allelic expression was observed, and in one of them IGF-2 expression too was bi-allelic (Figs. 1 and 2, patient 2). In the remaining two H19 informative cases H19 expression was mono-allelic, in one of them the IGF-2 expression was also mono-allelic (Figs. 1 and 2, patient 1).

3.2. H19 expression in bladder carcinoma derived cell lines

RNA from four human bladder cancer derived cell lines were examined by Northern blot hybridization in order to establish the level of H19 and IGF-2 expression during in vitro culturing. In three of them no expression of either H19 and IGF-2 could be detected (T24P; UM-UC-3; EJ 28) while in the HT-1376 cell line low levels of both H19 and IGF-2 transcripts were present (Northern blot data, not shown).

3.3. Tumor formation in nude mice, H19 and IGF-2 transcript levels in the xenograft

In order to investigate a possible correlation between the H19 and IGF-2 RNA levels and tumorigenesis induced by cells derived from bladder carcinoma, cells from these four bladder cancer cell lines were injected subcutaneously into nude mice. Tumors with a diameter of approximately 1 cm were observed after 3 weeks (UM-UC-3) or 5–6 weeks (T24P; EJ28; HT-1376).

We used RT-PCR to compare the level of H19 and IGF-2 transcripts in the cells before injection to that in the tumors and in the cells derived from these tumors and cultured in vitro for 3 passages. In the four bladder cancer cell lines a recurrent and consistent pattern in the relationship between H19 expression and tumorigenesis was obvious. Expression of the H19 gene was observed in tumors induced by cells with no detectable H19 transcript content before injection (Fig. 3A – lanes 1 and 2). The appearance of H19 RNA was restricted to the tumor formation process since after 3 passages of in vitro culturing of cells isolated from the tumor produced in nude mice, the H19 RNA level had decreased below the level of detection as in the cells before injection (Fig. 3A, lane 3). In order to investigate if this phenomenon is limited to the H19 gene we investigated additional genes for the following reasons. IGF-2 is a reciprocal

imprinted gene physically close to H19. The Ha-ras-1 gene expression because its expression is frequently increased in bladder carcinoma and the gene is located physically close to the H19 and IGF-2 genes on chromosome 11p15.5 [24] and the GAPDH gene, because it is a housekeeping gene which is expressed in every cell. The phenomenon described above (Fig. 3A) is specific for the H19 gene. From the data in Fig. 3B it is very clear that a consistent correlation between IGF-2 expression and tumorigeneity could not be observed. In total we determined the expression of H19 and IGF-2 genes in ten tumors and all of them confirmed the above mentioned conclusions. Also no changes in the expression of the Ha-ras-1 and the GAP-DH genes during tumorigenesis were observed (Fig. 3C,D).

In order to rule out the possibility that the RT-PCR product in the xenograft tumors is due to mouse H19 RNA, we performed RT-PCR with total RNA isolated from the C-2 myoblast mouse cell line, known to express the H19 gene [25]. No RT-PCR product was detected (Fig. 3, lane C-2).

The appearance of H19 RNA in the induced tumors may be accompanied by a loss of H19 imprinting. However the allelic pattern of the H19 expression in cells of the bladder carcinoma derived cell lines and in the tumors induced by them was not determined because *Rsa*I polymorphism was absent in the cell lines used in the experiments.

3.4. Localisation of H19 transcripts in the xenograft tumor

We further examined the xenograft tumor tissue, by in situ hybridization, in order to determine whether all tumor cells express increased levels of H19, or whether this phenomenon is restricted to certain cells only.

In the xenograft tumor sheets of pleomorphic cells are seen (Fig. 4A) and H19 is expressed in a number of small clusters and in single cells (Fig. 4B). This pattern of distribution is very similar to that observed in human bladder carcinoma in vivo [15].

4. Discussion

It has been claimed that the reciprocal imprinted genes H19 and IGF-2 are involved in the pathogenesis of embryonal tumors associated with the Beckwith-Wiedemann Syndrome [11] and other tumors such as testicular germ cell tumors [13] and lung cancer [14].

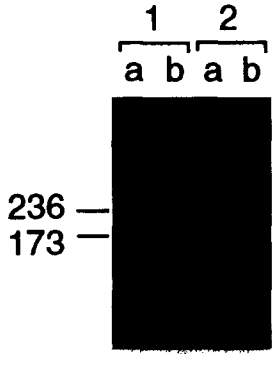

Fig. 2. Allelic mode of IGF-II expression in two cases of bladder with *Apa*I restriction polymorphism. Lanes a = RT-PCR products before and lanes b = after restriction with *Apa*I (the cases represented are identical to those in Fig. 1).

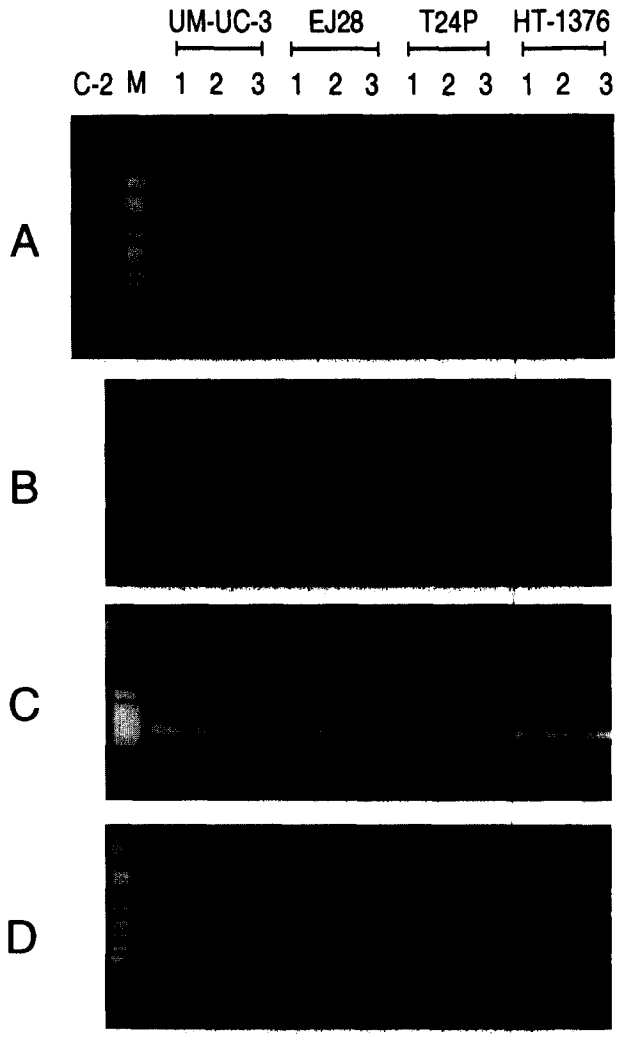

Fig. 3. Expression of H19, IGF-2, Ha-ras-1 and GAPDH in four bladder carcinoma cell lines and in tumors induced by them. Lanes 1 = cells before injection; Lanes 2 = tumors induced in nude mice; lanes 3 = cells derived from these tumors. Content of H19 (A); IGF-2 (B); Ha-ras-1 (C) and GAPDH (D) RNA determined by RT-PCR as described in section 2. Lane M = DNA molecular-weight marker VI (Boehringer-Mannheim). Lane C-2 = RT-PCR with total RNA from mouse myoblast cell line C-2.

In many cases Wilm's tumor is accompanied by the maternal loss of chromosome 11p15.5 and frequently paternal disomy of this chromosomal area was established [23]. These and similar findings formed the basis for the proposal that the H19 gene is a tumor suppressor gene. Further evidence for this proposal was provided by experiments carried out by Hao et al. [26] who introduced a construct expressing the H19 gene under the control of a metallothionein promoter into embryonal tumor cell lines. The transfected cells showed growth retardation but is noteworthy that in several of these cell lines the growth retardation occurred in cells with prominent structural changes. Suppression of tumorigeneity was shown for one of these cell lines. However, we have recently pointed out that in contradiction to its proposed tumor suppressor role, the H19 gene, is expressed in human bladder carcinoma of adults while the normal bladder tissue does not express the H19 gene [15].

We have further investigated the connection between H19

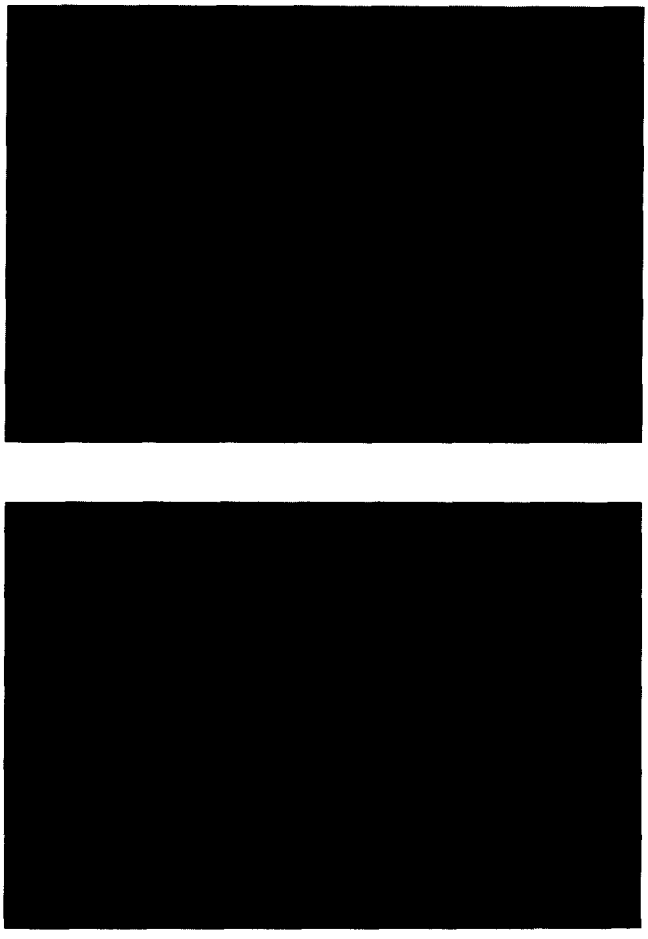

Fig. 4. Pattern of H19 RNA distribution in tumors induced by UM-UC-3 cells in nude mice. In situ hybridization with H19 [^{35}S]antisense RNA heamatoxylin-eosin as a counter stain. (A) Brightfield (×185); (B) the same as (A), but darkfield.

expression and tumorigenesis using cell lines from two different sources, a cell line derived from choriocarcinoma (JEG-3) and cell lines derived from human bladder carcinoma as described here.

H19 is expressed in cells of the choriocarcinoma derived cell line JEG-3. The level of the H19 transcripts in tumors formed after their injection into nude mice is 3–5 fold higher than in the original cells. The cells isolated from these tumors and cultured in vitro maintained the high level of H19 expression. We interpreted these results by assuming that cells with a relatively high level of H19 expression were selected during the process of tumorigenesis. In situ hybridization of the cells before injection and of the tumor with H19 antisense RNA supported this assumption [27].

No or very low expression of H19 was detected in the cells of four human bladder carcinoma cell lines in sharp contrast to the tumors induced by them in nude mice. However, cells isolated from these tumors had already lost their ability to express H19 after 3 passages of in vitro culture (Fig. 3A).

This observation excludes the possibility that the increase in the H19 RNA level in the tumor is due to the clonal expansion of a few cells in the tumor cell lines which express H19 at a comparatively high level. The spatial distribution pattern of the H19 expression in the xenograft (Fig. 4) supports this conclusion. It may, however, strongly indicate that H19 expression is restricted to bladder cancer cells at certain stages of tumor progression only, as seems to be the case according to our findings in bladder carcinoma in vivo [15].

The relationship between H19 expression and the development of tumors induced in nude mice by bladder carcinoma cell lines described here shows striking similarities to the process of tumorigenesis of bladder carcinoma in vivo. Human bladder carcinoma develops in the adult from bladder mucosa cells which have since long lost their ability to express the H19 gene, but H19 expression is activated during carcinogenesis of bladder carcinoma in vivo and during tumorigenesis induced by H19 non-expressing bladder carcinoma cells in nude mice. Also in both cases expression is likely limited to cells at certain stages of tumorigenesis only.

The very significant increase in the H19 transcript level during the process of tumorigenesis in vivo [15] and in vitro (in the nude mice as described above) may be the result of transcriptional activation and/or increase in the stabilization of the RNA. Also loss of imprinting of the gene, allowing transcription from two alleles, may be involved. The results described here clearly demonstrate that the loss of H19 imprinting can occur during the development of human bladder carcinoma in vivo but it cannot be solely responsible for the increase in the level of H19 transcripts because an increase is also detected in tumors expressing H19 in the mono-allelic mode (Fig. 1, lanes b). Also the loss of imprinting of the H19 and IGF-2 genes is not obligatory for the development of bladder carcinoma (Figs. 1 and 2). However, the possibility exists that biallelic expression of the H19 gene and/or the IGF-2 gene is a hallmark of those cells which are in advanced stages of tumorigenesis. In one of the tumors, both H19 and IGF-2 were bi-allelicly expressed, indicating uncoupling of H19 and IGF-2 expression in an adult tumor. A similar finding was recently reported in a case of juvenile hepatoblastoma [28].

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