hH-Rev107, a class II tumor suppressor gene, is expressed by post-meiotic testicular germ cells and CIS cells but not by human testicular germ cell tumors.

Sylvie Siegrist, Chloé Féral, Mounia Chami, Brigitte Solhonne, Marie-Geneviève Mattei, Ewa Rajpert-de Meyts, Georges Guellaën, Frédérique Bulle

h*H-Rev107*, a class II tumor suppressor gene, is expressed by post-meiotic testicular germ cells and CIS cells but not by human testicular germ cell tumors

Sylvie Siegrist[1] , Chloé Féral[1], Mounia Chami[1], Brigitte Solhonne[1], Marie-Geneviève Mattéi [2], Ewa Rajpert-De Meyts[3], Georges Guelläen[1], and Frédérique Bulle*[1]

[1] INSERM U99, Hôpital H. Mondor, 94010 Créteil, France.

[2] INSERM U491, Faculté de Médecine, 13385 Marseille, France.

[3] Copenhagen University, Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark.

* Corresponding author :

Tel.: (33) 1 49 81 25 40

Fax: (33) 1 48 98 09 08

E-mail: bulle@im3.inserm.fr

Running title: h*H-Rev107* in human testicular cancer

Keywords: h*H-Rev107*; tumor suppressor gene; spermatogenesis; carcinoma in situ; testicular germ cell tumors.

Abstract

By systematic analysis of a human testis library, we have isolated the h*H-Rev107-3* cDNA, identical to h*H-Rev107-1* cDNA, which was previously described as a class II tumor suppressor gene (Husmann et al., 1998). In this study, two transcripts (1 and 0,8 kb) were detected by Northern blot in all human tissues, excepted in thymus. The strongest expression was found in testis, skeletal muscle and heart. These two mRNA are probably transcribed from only one gene that we mapped to the q12-q13 region of the chromosome 11. In human testis, h*H-Rev107* gene expression was localized, by in situ hybridization, within the round spermatids. To investigate a possible role for hH-Rev107 protein in testicular malignant growth, we examined the expression of this gene in germ cell tumors. A strong h*H-Rev107* gene expression was observed in normal testis as well as in samples with preinvasive carcinoma in situ but was completely absent in overt tumors, both seminomas and non-seminomas. By in situ hybridization, CIS was found h*H-Rev107* positive and tumor negative. A semi-quantitative assessment of h*H-Rev107* mRNA level in testicular germ cell tumors, by RT-PCR, exhibited a 9-fold decrease in the gene expression. No gross structural aberrations of h*H-Rev107* gene were detected in these human primary tumors. The results suggest that down-regulation of h*H-Rev107* may be associated with invasive progression of testicular germ cell tumors.

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

Introduction

Testicular tumors are rare, comprising approximately 2% of all cancers occurring in men. However, this neoplasia is the most common malignant disease in males aged 15-35 years (Richie, 1993) and represents the most frequent cause of death from solid tumors in this patient group. The incidence has steadily risen in the past 30-50 years, particularly in the western world (Peckham, 1988) (Schmidt et al., 1995).

The large majority of testicular tumors originate from germ cells, rarely from Sertoli or Leydig cells. Testicular germ cell tumors (TGCTs) are a heterogeneous group of different histological tumors which are divided into two major groups: seminomas, which proliferate along germ cell lineage, and non-seminomas, which display embryonal differentiation and exhibit embryonal (embryonal carcinoma), extra-embryonal (choriocarcinoma, yolk sac tumor), and somatic (teratoma) patterns of tissue differentiation. Most of TGCTs are derived from a common preinvasive precursor, carcinoma in situ (CIS) cells, which may represent gonocytes that have been delayed in their progression to spermatogonia (Skakkebaek, 1972) (Skakkebaek et al., 1987). Because the molecular pathogenesis of germ cell tumors is not known, identification of genes involved in testicular tumorigenesis will increase our understanding of development of testicular tumors and may provide the basis for new-targeted therapies.

By systematic sequence analysis of cDNAs expressed in human testis, we isolated new human transcripts (Pawlak et al., 1995), some of which correspond to genes that are potentially involved in cell division. Among them, one human testis cDNA sequence was found to be identical to the human *H-Rev107-1* cDNA (Husmann et al., 1998), which was proposed to act as a class II tumor suppressor gene.

The r*H-Rev107* cDNA was first isolated in rat cells from a phenotypic revertant (F9 cells) of *H-Ras* transformed rat fibroblasts (FE-8 cells). No r*H-Rev107* transcript was detected in FE-8 cells. Apart from F9 cells, a strong expression of the r*H-Rev107* gene was also found in a fibroblast line which is resistant to transformation by *H-Ras*, suggesting that the r*H-Rev107* gene was directly involved in the resistance toward *H-Ras* mediated transformation (Hajnal et al., 1994). Further studies have shown that the r*H-Rev107* gene was frequently down regulated in rat tumorigenic cell lines and in experimental tumors mediated by targeted expression of a *H-Ras* transgene. Additionally, forced expression of the r*H-Rev107* cDNA, controlled by heterologous promoters in deficient rodent cell lines transformed by *H-Ras*, resulted in growth inhibition in vivo and in vitro (Sers et al., 1997). As repression of the *H-Rev107* gene occurred within the tumor cell lines without any modification at the DNA level, the *H-Rev107* gene was categorised as a class II tumor suppressor gene (Sers et al., 1997). This class of antioncogenes possesses a functionally intact gene, but its expression is repressed in tumors, and permits cell proliferation (Lee et al., 1991). The human orthologue of r*H-Rev107* cDNA (h*H-Rev107-1*) was also shown to be down regulated in tumorigenic cell lines both at the level of transcription and translation (Husmann et al., 1998).

Given the evidence for the expression of h*H-Rev107* gene in human testis and the antiproliferative function of the encoded protein, we wished to explore the potential role of H-Rev107 protein in human testicular germ cell pathogenesis. In this study, we analyzed the expression of the h*H-Rev107* gene in human tissues; we localized the transcript in human testis within the germ cells, in CIS but not in TGCTs. We quantified gene expression by RT-PCR in a series of human TGCTs compared with normal testis and with the preinvasive CIS. Our data identify hH-Rev107 as

candidate novel marker associated with progression of human TGCTs from preinvasive to invasive lesions.

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

Results

Cloning and sequencing of h*H-Rev107-3 cDNA*

We cloned a human testis cDNA (h*H-Rev107-3*) (Accession number: AF317086) which exhibited 99,4% identity with the h*H-Rev107-1* cDNA (Husmann et al., 1998) with two insertions in the 5' and 3' non coding sequences and two mutations in the coding sequence leading to serine versus threonine. Sequences of 354 bp and 42 bp were missing in 5' and 3', respectively. The presence of a poly(A)+ tail at the 3' end of h*H-Rev107-3* cDNA indicates that the transcript cloned from the testis has used an alternative polyadenylation site (Figure 1).

Expression of hH-Rev107 gene in human tissues

The distribution of h*H-Rev107* mRNA in human tissues revealed one or two transcripts of 1 and 0,8 kb from all tissues analyzed, apart from thymus where no signal was detected (Figure 2). The strongest expression was found in testis, skeletal muscle and heart. The same tissue distribution was reported by Husmann (Husmann et al., 1998) but the presence of two messengers were previously unnoticed, probably due to an overexposure of the blots. The relative distribution of these two mRNA was quantified from a weak exposure (data not shown) and was found tissue-specific: the 0,8 kb mRNA was only detected in leucocytes and spleen, and was preferentially expressed in pancreas, skeletal muscle and heart; the 1 kb mRNA was mainly expressed in testis, liver and brain. In mouse, only one messenger of 1 kb was detected by Northern blot of mouse tissues probed with h*H-Rev107-3* cDNA, except in testis where two messengers were present (1 and 0,8 kb) (data not shown).

Analysis of human genomic DNA

Human genomic DNA was digested using restriction enzymes cutting only once (*Pst* I) or none (*Bam*H I, *Kpn* I, *Eco*R I and *Hin*d III) in the h*H-Rev107-3* cDNA sequence. Southern blot, hybridized with h*H-Rev107-3* probe, revealed a simple pattern of 3 to 5 bands, leading to a total of 16 to 40 kb for the h*H-Rev107* gene, that led us to conclusion that only one gene for h*H-Rev107* exists in the human genome (Figure 3).

h*H-Rev 107* *chromosomal localization*

In the 100 metaphase cells examined after in situ hybridization, 26.6% of silver grains were located on chromosome 11; 80% of them mapped to the q12-q13.2 region chromosome 11 long arm. These results allow us to localize the gene h*H-Rev 107* to the 11 q12-q13.2 region of the human genome (Figure 4). A similar result was obtained by Husmann (Husmann et al., 1998), on chromosome 11 q11-12, by FISH.

Human EST database analysis

The h*H-Rev107-3* cDNA nucleotide sequences were searched for homology in the dbEST database using the BLAST program. We only detected EST sequences that were 100% identical to h*H-Rev107-3* cDNA, no mutated sequence; this validates the sequence of h*H-Rev107-3*. From 3' end EST analysis, we found an EST population produced from different human tissues, similar to the end of h*H-Rev107-3* cDNA. No spliced mRNA was found from EST analysis. Taken together, these results indicated the presence of only one gene for h*H-Rev107* in the genome and the two mRNA are likely transcribed from two different promoters with the use of two polyadenylation sites.

Hal-Inserm Author manuscript

Localization of h<u>H-Rev107</u> gene expression in human testis

Detection of h*<u>H-Rev107</u>* transcripts was further characterized in human testis by in situ hybridization of DIG-labelled h*<u>H-Rev107</u>* antisense oligonucleotide (Figure 5, B and D). The presence of the h*<u>H-Rev107</u>* mRNA was confined to the seminiferous tubules in association with germ cells and more precisely in round spermatids. In situ hybridization of DIG-labelled h*<u>H-Rev107</u>* sense oligonucleotide to human testis showed no staining (Figure 5, A and C).

Expression of h<u>H-Rev107</u> gene in CIS and testis tumors

We analyzed, by Northern blot, the h*<u>H-Rev107</u>* mRNA level in a panel of 4 human TGCTs (2 seminomas and 2 non-seminomas), 2 pure CIS and 4 normal testes (Figure 6) (Table 1). A strong expression of 1 and 0,8 kb mRNA was detected in the four normal testis samples as well as in the two CIS. In contrast, there was a lack of expression of these two h*<u>H-Rev107</u>* transcripts in all the TGCTs tested. No significant variation in the amount of loaded mRNA was detected with the ß-actin control probe (data not shown).

To assess the localization of h*<u>H-Rev107</u>* expression in CIS and to verify the lack of signal in tumor, we performed an in situ hybridization of a DIG-labeled h*<u>H-Rev107</u>* antisense oligonucleotide on two CIS and one seminoma (Table 1). The presence of the h*<u>H-Rev107</u>* mRNA was detected within tubules in association with germ cells of the CIS (Figure 7, E, F and G). No signal was detected in seminoma (Figure 7, H). In situ hybridization of DIG-labelled h*<u>H-Rev107</u>* sense oligonucleotide to CIS and tumor showed no staining (Figure 7, A-D).

Hal-Inserm Author manuscript

In order to quantify the decrease of the h*H-Rev107* mRNA level occurring in testicular tumors, we performed a semi-quantitative RT-PCR on 16 TGCTs: 6 non-seminomas, 5 seminomas and 5 combined tumors (mixed seminoma and non-seminoma in the same sample) and compared the result with surrounding tissues (Table 1). A unique PCR product, with the expected size of 571 bp, was obtained suggesting that the two h*H-Rev107* mRNA have a common sequence between the positions 319 and 890 (referred to accession number X92814). This result is in agreement with the hypothesis of two mRNA transcribed from two different promoters with the use of two polyadenylation sites. RT-PCR of ß2 microglobulin was performed in parallel as a control of the amount of starting mRNA. The relative h*H-Rev107* / ß2 microglobulin ratio has been estimated on the mean of three independent experiments. In all tumors tested, we observed a decrease of the h*H-Rev107* mRNA level, as compared to the surrounding tissues (Figure 8). It is noteworthy that surrounding tissues exhibit a great heterogeneity based on histology results and can be classified in three groups: group 1, corresponding to normal tissues (N); group 2, corresponding to normal tissues containing CIS (N +%CIS) and group 3, corresponding to CIS with variable amount of tumor (CIS +%T) (Table 1). We observed a 9-fold decrease of the h*H-Rev107* mRNA level between the group 1 (mean = 58; SE = 21) and the group of TGCTs (mean= 6,18; SE = 7,74), which is statistically significant (P value = 0,0021) (Mann-Whitney test). It is worth mentioning that there is no difference (P = 1) between the group 1 (mean = 58; SE = 21) and the group 2 (mean = 53,4; SE = 8,8) whatever the percentage of CIS present in the parenchyma tissue. This result is in good agreement with the h*H-Rev107* strong signal observed in pure CIS by Northern blot and by in situ hybridization. On the contrary, we observed a statistical difference (P = 0,038) between the group 1 (mean = 58; SE = 21) and 3 (mean = 14,16; SE = 3,3) that most likely reflects the presence of tumor tissues in these CIS.

Hal-Inserm Author manuscript

Discussion

In this study, we have cloned a human testis h*H-Rev107-3* cDNA and provided evidences indicating that only one h*H-Rev107* gene is present in the human genome and encodes two mRNA. From the results of sequencing, EST database analysis and RT-PCR, it is inferred that the two mRNA are likely transcribed from two promoters with the use of two polyadenylation sites. As ESTs identical to h*H-Rev107-3* cDNA 3' end were obtained from different tissues, the use of the 3' polyadenylation site found in h*H-Rev107-3* cDNA is not testis-specific. The deduced protein sequence did not exhibit a particular motif, which would be relevant to a specific biochemical function.

The physiological antiproliferative and tumor-suppressing activities of the Hrev-107 protein were demonstrated by r*H-Rev107* over expression in *H-Ras* transformed cells in vitro and in vivo (Sers et al., 1997). In accordance with these observations, we have attempted to analyze the expression of h*H-Rev107* gene in normal human testis and diverse types of testicular tumors. Given the unique features of this natural model system for simultaneous analyses of human spermatogenesis, cell division and differentiation and tumorigenesis (Skakkebaek et al., 1987), we shall consider our present findings in relation to the diverse patterns of differentiation, and then discuss their potential implications for the pathogenesis of testicular cancer.

The process of germ cell differentiation begins in embryo when primordial germ cells differentiate into gonocytes, which subsequently mature into spermatogonia during infancy and early childhood. At the onset of puberty, gonadotropic stimulation induces real spermatogenesis. Using in situ hybridization, we localized h*H-Rev107* mRNA in the round spermatids in human testis. These cells belong to the differentiation phase just before the condensation of the nuclei

occurring in elongating spermatids. The proteins translated just after meiosis correspond to a limited number of genes that are involved in the final differentiation of spermatozoon (Erickson, 1990) (Schmidt & Schibler, 1995) (Hecht, 1998) (Braun, 1998). The post-meiotic expression of h*H-Rev107* gene is still unclear, but the restricted expression pattern of h*H-Rev107* mRNA in round spermatids is consistent with a specialized role during spermiogenesis. This hypothesis is in good agreement with the putative function of H-Rev107 protein, which is to suppress cell proliferation and / or to maintain the cell in a differentiated state (Husmann et al., 1998).

Several studies have provided pieces of evidence in line with the hypothesis that inhibition of gonocyte differentiation into spermatogonia during fetal life or infantile period may be crucial for the development of CIS (Skakkebaek et al., 1987) (Rajpert-De Meyts et al., 1998). CIS cells may differentiate, in the young adulthood along pathways resulting in several histological tumor types such as seminoma and non-seminoma. Using in situ hybridization and semi-quantitative RT-PCR, we demonstrated the presence of h*H-Rev107* mRNA in preinvasive CIS but not in overt tumors (seminoma, non-seminoma and combined tumor). This result is in favor of the putative role for H-Rev107 protein in control of cell division, thus its absence may permit a proliferative process. There are very few reliable molecular markers that correlate closely with progression of the TGCTs. Among the cell cycle regulators studied in testicular cancer, $p18^{INK4C}$, a CDK inhibitor and candidate tumor suppressor, has the same pattern of expression in TGCTs and CIS as h*H-Rev107* gene. But its expression, within normal tubules, is not restricted to a specific phase of spermatogenesis; (Bartkova et al., 2000). Due to its specific expression in the normal testis, H-Rev107 protein could be considered as a candidate regulator potentially involved in the process of post-meiotic maturation stages of human spermatogenesis.

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

The relative down-regulation of h*H-Rev107* gene in TGCTs in comparison to CIS might serve as indicator of progression of tumorigenesis.

All studies of H-Rev107 in rat or in human demonstrated an inverse correlation between *H-Rev107* and the *H-Ras* gene expressions (Hajnal et al., 1994; Husmann et al., 1998). It is well documented that Ras plays a role in the normal cell growth differentiation and activated Ras oncogenes have been identified in a wide variety of human malignancies (Bos, 1989). In testis tumors, no mutation was observed in the coding sequence of *H-Ras* and no significant variation was observed in the mRNA level of *H-Ras* in seminoma as well as embryonal carcinoma or teratoma (Mulder et al., 1989) (Moul et al., 1992) (Shuin et al., 1994). We performed semi-quantative RT-PCR for *H-Ras* on a small series of tumors with their surrounding tissues. No evident correlation could be demonstrated between h*H-Rev107* and *H-Ras* gene expression (data not shown).

To explain the loss of the h*H-Rev107* mRNA in TGCTs, we examined if there were genomic rearrangements in DNA from human primary tumors. No difference was observed when comparing Southern blot profiles of DNA obtained from human testis tumor tissues and from human blood (data not shown). As h*H-Rev107* mRNA was down-regulated in human testis tumors without gross alteration at the DNA level, the loss of expression may be due to: i) a limited genomic modification (mutation or small deletion) in the promoter region; ii) the absence of regulatory protein which is upstream in the cascade of cell proliferation pathway. Because repression of the r*H-Rev107* gene occurred within the tumor cell lines without any modification at the DNA level, Sers considered r*H-Rev107* as belonging to the class II tumor suppressor gene (Sers et al., 1997). The concept of class II tumor suppressor gene was first developed by Lee (Lee et al., 1991) when loss of function is caused by a

regulatory block to expression. The study of this type of gene is of clinical interest as such a gene may theoretically be up regulated by drugs or special treatments.

A search for homology in the GenBank database revealed that *H-Rev107* might be a member of a gene family. The second member would be the retinoic acid receptor responder 3 gene (*RARRE3*, also called *TIG3*) (DiSepio et al., 1998), which was also cloned as the retinoid-inducible gene (*RIG1*) (Huang et al., 2000) and as h*H-Rev107-2* (Husmann et al., 1998). The nucleotide sequence of this second member is 54% identical to h*H-Rev107* gene. The tissue expression pattern and the gene regulation of *TIG3/RIG1* gene are different from the one of h*H-Rev107* gene (DiSepio et al., 1998) (Huang et al., 2000) But *TIG3* would also have an anti-proliferative function and may function as a tumor suppressor in both normal and malignant epidermal differentiation (DiSepio et al., 1998) (Duvic et al., 2000).

The gene family is localized on the same chromosome 11 in 11q12 region for h*H-Rev107* gene (this work, (Husmann et al., 1998)) and 11q23 region for *RARRE* gene (DiSepio et al., 1998). The distribution of genes along 11q is non-uniform. Three regions of long arm of this chromosome, 11q13, 11q22-23 and 11q24 have a higher density of genes. The reasons for this clustering are not completely explained, though an ancestral pericentric inversion juxtapositioning the gene-rich telomeric part of the centromere to its current location at 11q13 has been invoked (Saccone et al., 1992). Since h*H-Rev107* and *RARRE* genes are localized on the same region, they are likely to be related genes coming from the same ancestral sequence duplicated on this chromosome.

H-Rev107 gene belongs to a gene family, which plays a role in the control of cell proliferation. It would be important to determine exactly the regulatory pathways in which these genes are involved. This could improve our understanding of cell proliferation in tumorigenesis. In addition, analysis of their promotor regions

Hal-Inserm Author manuscript

may contribute in the development of new drugs that can induce h*H-Rev107* expression and thereby exert an antiproliferative effect.

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

Materials and methods

Tissues, genomic DNA and RNA preparation

Small specimens (0.1 to 2 mg) of human testicular tumors and surrounding tissues were obtained at the time of surgery from patients undergoing orchidectomy. Immediately after macroscopic evaluations, tissues were frozen at -80°C. Histological evaluation was based on routine pathology reports and included seminomas, non-seminomas, mixed germ cell tumors (seminoma and non-seminoma) and CIS. Normal testicular tissues were obtained from patients undergoing bilateral orchidectomy for treatment of prostatic carcinoma (Table 1).

Genomic DNA was obtained from peripheral blood cells and prepared as previously described (Bulle et al., 1996).

Total RNA was isolated from frozen tissue specimens using the method of Chomczynski (Chomczynski & Sacchi, 1987).

cDNA cloning and sequencing

The h*H-Rev107-3* cDNA (Accession number: AF317086) was isolated from a human testis library and the complete sequence of the cDNA (676 bp) was determined as previously described (Pawlak et al., 1995).

Northern and Southern blot analysis

Human multiple-tissue Northern blots were purchased from Clontech (Palo Alto, Calif.). Poly(A)+ RNA from different human tumor tissues were analyzed by Northern blot as previously described (Levy et al., 1996).

20 µg of genomic DNA were digested by *Bam*H I, *Kpn* I, *Pst* I, *Eco*R I and *Hin*d III. The fragments were separated on 0.8% agarose gel and blotted onto Hybond N membrane (Amersham) as previously described (Bulle et al., 1996).

The blots were hybridized with the h*H-Rev107-3* or β-actin cDNA (Accession number: NP 001092) sequences labelled by random priming, with a ^{32}P dCTP, according to the Megaprime protocol (Amersham).

Northern blots were washed in 0.05 X SCC, 0.1% SDS at 65°C and Southern blots in 0.5 X SCC, 0.1% SDS at 68°C.

Chromosomal localization

In situ hybridization was carried out on chromosome preparations obtained from phytohemagglutini-stimulated lymphocytes and hybridized with the tritium labelled h*H-Rev107*-3 clone (Mattei et al., 1985). The slides were exposed for 20 days at chromosome spreads were first stained with buffered giemsa solution and metaphases photographed. R-banding was then performed by the fluorescence-photolysis-giemsa (F.P.G.) method and metaphases rephotographed before analysis.

In situ hybridization

In situ hybridization was carried out on sections of paraffin-embedded testis. After deparaffinization in xylene and rehydratation, sections were incubated in 50% acetic acid for 30 sec, and then rinsed in sterile water. Sections were digested with 10 µg / mL proteinase K in 100 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$ for 15 min at 37°C. Slides were then washed in sterile water, and postfixed in 2% paraformaldehyde (PFA) for 20 min at room temperature. After one wash in sterile water, sections were dehydrated in gradual ethanol baths. Following air-dry treatment, sections were prehybridized for 2 h at 37 °C in 100 µL of hybridization buffer (4% deionized formamide, 1 X

Hal-Inserm Author manuscript

Denhardt's solution, 1.5 X SSC and 330 µg / mL yeast tRNA) in a humid chamber. In situ hybridization was performed for 16 h at 37 °C in 20 µL of the hybridization buffer containing 10% dextran sulfate and 25 ng DIG-labeled oligonucleotide antisense to the h*H-Rev107* mRNA (position 571-598, accession number: X92814) in a humid chamber. After hybridization, the sections were briefly rinsed in 2 X SSC at room temperature, then in 2 X SSC for 1 h at 37°C, and finally in 1 X SSC for 30 min at room temperature. Hybridization signals were detected using DIG Nucleic Acid Detection Kit (Boehringer Mannheim) with alkaline phosphatase-conjugated anti-DIG antibody, nitroblue tetrazolium salt / bromo-chloro-indolyl-phosphate (NBT / BCIP) as chromogen, and high molecular weight polyvinyl alcohol (10%). Sections were then counterstained using methyl green. Specificity experiments included hybridization with the DIG-labelled sense oligonucleotide.

Semi-quantitative RT-PCR analysis

Amplification of h*H-Rev107* mRNA was performed from primers, which map at position 320-345 (5'-TTAATGACAATAAATCCCTGCTCCCC-3') and 869-890 (5'-CTCAAGAAACAAGCGACAAAAG-3') (Accession number: X92814) and generates a 571 bp fragment. Primers used for the amplification of ß2 microglobulin mRNA (Accession number: XM_007650) map at position 11-36 (5'-GAGATGTCTCGCTCCGTGGCCTTAGC-3') and 358-385 (5'-GGATCGAGACATGTAAGCAGCATCATGG-3') and generate a 375 bp fragment. All primers were synthesized commercially (Genset, France). RT-PCR reactions were carried out with 5 or 1 ng of total RNA for h*H-Rev107* or ß2 microglobulin amplification, respectively using the RT-PCR access kit (Promega). RT-PCR was performed by using 30 or 40 amplification cycles in a thermal cycler (Perkin Elmer) at an annealing temperature of 54°C or 58°C, respectively. The PCR products were

Hal-Inserm Author manuscript

separated on a 2% agarose gel and transferred onto Hybond N membrane (Amersham). The specific products were revealed by hybridization to a ^{32}P oligonucleotide probe specific of h*H-Rev107* mRNA (5'-CTGACTGACAAGGCCATCGTG-3') (nt 567-587) or ß2 microglobulin (5'-GAAGTTGACTTACTGAATGGAGAG-3') (nt 179-203). The signal was quantified in an Instant Imager using the Imager program. The amount of RNA, the number of amplification cycles and the conditions of exposure were selected in order to obtain a signal in a linear concentration range.

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

Acknowledgements

We thank Y. Laperche for helpful discussion and J. Hinrichsen, H. Kistrup and C. Deschamps for excellent technical assistances. INSERM and the Danish Cancer Society funded this work.

Hal-Inserm Author manuscript

References

Bartkova, J., Thullberg, M., Rajpert-De Meyts, E., Skakkebaek, N.E. & Bartek, J. (2000). *Int J Cancer*, **85,** 370-5.

Bos, J.L. (1989). *Cancer Res*, **49,** 4682-9.

Braun, R.E. (1998). *Semin Cell Dev Biol*, **9,** 483-9.

Bulle, F., Lespinasse, J., Pawlak, A., Vadot, E., Sastre, J., Noel, B. & Guellaen, G. (1996). *Ann Genet*, **39,** 139-43.

Chomczynski, P. & Sacchi, N. (1987). *Anal Biochem*, **162,** 156-9.

DiSepio, D., Ghosn, C., Eckert, R.L., Deucher, A., Robinson, N., Duvic, M., Chandraratna, R.A. & Nagpal, S. (1998). *Proc Natl Acad Sci U S A*, **95,** 14811-5.

Duvic, M., Helekar, B., Schulz, C., Cho, M., DiSepio, D., Hager, C., DiMao, D., Hazarika, P., Jackson, B., Breuer-McHam, J., Young, J., Clayman, G., Lippman, S.M., Chandraratna, R.A., Robinson, N.A., Deucher, A., Eckert, R.L. & Nagpal, S. (2000). *Clin Cancer Res*, **6,** 3249-59.

Erickson, R.P. (1990). *Trends Genet*, **6,** 264-9.

Hajnal, A., Klemenz, R. & Schafer, R. (1994). *Oncogene*, **9,** 479-90.

Hecht, N.B. (1998). *Bioessays*, **20,** 555-61.

Huang, S.L., Shyu, R.Y., Yeh, M.Y. & Jiang, S.Y. (2000). *Mol Cell Endocrinol*, **159,** 15-24.

Husmann, K., Sers, C., Fietze, E., Mincheva, A., Lichter, P. & Schafer, R. (1998). *Oncogene*, **17,** 1305-12.

Lee, S.W., Tomasetto, C. & Sager, R. (1991). *Proc Natl Acad Sci U S A*, **88,** 2825-9.

Levy, I., Wu, Y.Q., Roeckel, N., Bulle, F., Pawlak, A., Siegrist, S., Mattei, M.G. & Guellaen, G. (1996). *FEBS Lett*, **382,** 276-80.

Mattei, M.G., Philip, N., Passage, E., Moisan, J.P., Mandel, J.L. & Mattei, J.F. (1985). *Hum Genet*, **69,** 268-71.

Moul, J.W., Theune, S.M. & Chang, E.H. (1992). *Genes Chromosomes Cancer*, **5,** 109-18.

Mulder, M.P., Keijzer, W., Verkerk, A., Boot, A.J., Prins, M.E., Splinter, T.A. & Bos, J.L. (1989). *Oncogene*, **4,** 1345-51.

Pawlak, A., Toussaint, C., Levy, I., Bulle, F., Poyard, M., Barouki, R. & Guellaen, G. (1995). *Genomics*, **26,** 151-8.

Peckham, M. (1988). *Acta Oncol*, **27,** 439-53.

Rajpert-De Meyts, E., Jorgensen, N., Brondum-Nielsen, K., Muller, J. & Skakkebaek, N.E. (1998). *Apmis*, **106,** 198-204; discussion 204-6.

Richie, J.P. (1993). *CA Cancer J Clin*, **43,** 151-75.

Saccone, S., De Sario, A., Della Valle, G. & Bernardi, G. (1992). *Proc Natl Acad Sci U S A*, **89,** 4913-7.

Schmidt, B., Ackermann, R. & Strohmeyer, T. (1995). *J Mol Med*, **73,** 355-67.

Schmidt, E.E. & Schibler, U. (1995). *Development*, **121,** 2373-83.

Sers, C., Emmenegger, U., Husmann, K., Bucher, K., Andres, A.C. & Schafer, R. (1997). *J Cell Biol*, **136,** 935-44.

Shuin, T., Misaki, H., Kubota, Y., Yao, M. & Hosaka, M. (1994). *Cancer*, **73,** 1721-7.

Skakkebaek, N.E. (1972). *Lancet*, **2,** 516-7.

Skakkebaek, N.E., Berthelsen, J.G., Giwercman, A. & Muller, J. (1987). *Int J Androl*, **10,** 19-28.

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

Legends to figures

Table 1: Description of the testis samples.

The samples are identified by their tissue numbers, (I-macroscopically tumor; II-macroscopically surrounding tissues), the diagnosis and the histology.

Figure 1: Sequence of the human h*H-Rev107-3* cDNA.

Nucleotide sequence of the human h*H-Rev107-3* cDNA (Accession number: AF317086) and the predicted amino acid sequence. As compared to h*H-Rev107-1* sequence (Accession number: X92814), the two inserted nucleotides are represented in bold and the two mutated nucleotides are boxed.

Figure 2: Expression of h*H-Rev107* gene in human tissues

Northern blot analysis was performed using human multiple-tissue blots containing 2 µg of poly(A)+ RNA per lane, and hybridized with ^{32}P labeled h*H-Rev107-3* cDNA. Arrows on the right indicate the size of the mRNA.

Figure 3: Analysis of human genomic DNA

Southern blot was performed with human genomic DNA digested with the indicated restriction enzymes, and hybridized with ^{32}P labeled h*H-Rev107-3* cDNA. The blot was exposed 7 nights with two screens. The size of the markers is indicated on the left.

Figure 4: Chromosomal localization of the human gene h*H-Rev107*

Idiogram of the human G-banded chromosome 11 illustrates the distribution of labelled sites with the h*H-Rev107–3* probe

Figure 5: Localization of h*H-Rev107* gene expression in normal human testis

Paraffin-embedded testis sections (tissue number c3, Table 1) were hybridized with: **A and C**, h*H-Rev107-3* sense DIG-oligonucleotide, **B and D**, h*H-Rev107-3* antisense DIG-oligonucleotide. Sections were counterstained with methyl green. Original magnifications 200 X (A and B), 630 X (C and D). Arrows indicate signal.

Figure 6: Expression of h*H-Rev107* gene in testis tumors

Northern blot analysis was performed with 3 µg of poly(A)+ RNA per lane isolated from 4 normal testes (N), 2 carcinoma in situ (CIS), 2 non-seminoma (NSE) and 2 seminoma (SE). 4789-IIA to 4808-I corresponds to the tissue numbers described in Table 1. The blot was hybridized with ^{32}P labeled h*H-Rev107-3* cDNA. Arrows on the left indicate the size of the mRNA.

Figure 7: Localization of h*H-Rev107* gene expression in human CIS and testis tumor.

Paraffin-embedded testis sections were hybridized with the h*H-Rev107-3* sense DIG-oligonucleotide (**A, B, C and D**) or the antisense h*H-Rev107-3* DIG-oligonucleotide. (**E, F, G and H**). Sections were counterstained with methyl green. Original magnifications 200 X. **A**, **E**: CIS, tissue number 5294-II, **B-G**: CIS, tissue number 5423-II, **D, H**: seminoma, tissue number 5327-II (Table 1).

Figure 8: Semi-quantitative analysis of h*H-Rev107* gene expression in testis tumors.

The quantitative h*H-Rev107* and ß2 microglobulin gene expression was performed by RT-PCR on total RNA extracted from surrounding testicular tissues: 3 normal testicular tissues (), 6 normal tissues with increasing amounts of CIS () and 6 CIS with variable amounts of tumors () and from testicular tumors: 5 seminomas (), 6 non-seminomas () and 5 combined tumor (, samples are identified by their

number, I-macroscopically tumor; II-macroscopically surrounding tissues, (Table 1). The PCR products were revealed after blotting and hybridization with specific ^{32}P oligonucleotide. The h*H-Rev107* / ß2 microglobulin gene expression ratio is referred to the normal sample 1575 (in percentage). The results are based on the mean of three independent experiments.

Hal-Inserm Author manuscript

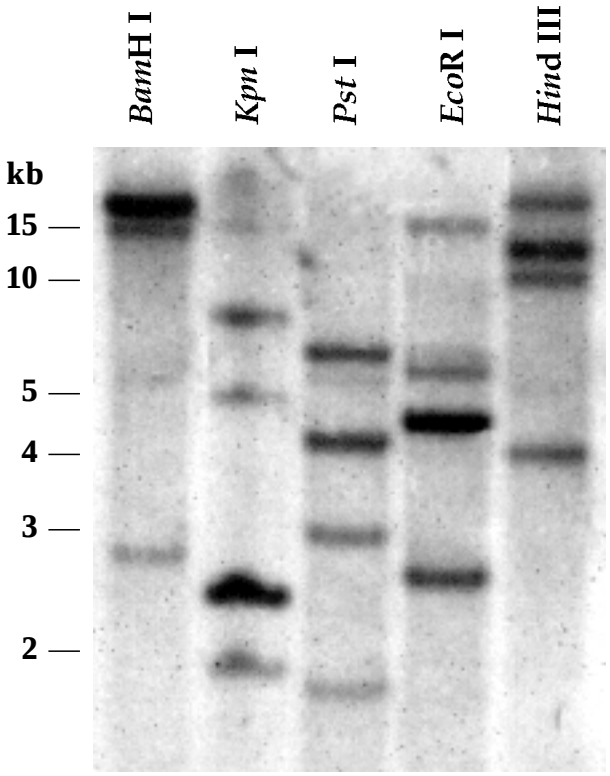
Tissue number	Diagnosis	Histology of testis
ISH		
c3	normal	
5423-II	CIS	CIS (80 %) + tubules with spermatogenesis (20 %)
5294-II	CIS	CIS (50 %) + tubules with spermatogenesis (50 %)
5327-II	Seminoma	Seminoma (30 %) + tubules with spermatogenesis (70 %)
Northern blot		
4789-IIA	normal	
4800	normal	
4749	normal	atrophic testis, few tubules with spermatogenesis
4789-IIB	normal	
4825-II	CIS	CIS (100 %)
4818	CIS	CIS (100 %)
4846	Non-Seminoma	Embryonal Carcinoma
4825-I	Non-Seminoma	Teratoma + Endodermal Sinus Tumor
4464-I	Seminoma	sample may contain a few CIS tubules
4808-I	Seminoma	
RT-PCR		
1575	normal	
3522-II	normal	
4084-II	normal	partly atrophic testis
4232-II	CIS	CIS (20 %) + normal or atrophic tubules (80%)
3769-II	CIS	CIS (50 %) + normal (50 %)
4227-II	CIS	CIS (50 %) + normal spermatogenesis (50%) ; may also contain tumor
4237-II	CIS	CIS (50 %) + normal (50 %) ; may also contain tumor
4296-II	CIS	CIS (80-90 %) + normal (10-20 %)
5423-II	CIS	CIS (90-100 %) + normal (10 %)
4134-II	CIS	CIS ; no spermatogenesis seen, may also contain Seminoma
4217-II	CIS	CIS (20-30%) ; may also contain Seminoma, the rest is partly atrophic
4037-II	CIS	CIS (30 %) ; may contain tumor, no spermatogenesis seen
5220-II	CIS	CIS (50-70 %) ; may contain foci of Seminoma, no spermatogenesis seen
5304-II	CIS	CIS (100 %) ; no spermatogenesis seen
4332-II	CIS	CIS (100 %) ; no spermatogenesis seen, but may also contain some tumor
4084-I	Non-Seminoma	Teratoma
4296-IA	Non-Seminoma	Teratoma
4227-I	Non-Seminoma	Teratoma + Embryonal Carcinoma
4296-IB	Non-Seminoma	Teratoma + Embryonal Carcinoma
5239-I	Non-Seminoma	Teratoma + Embryonal Carcinoma
5303-I	Non-Seminoma	Embryonal Carcinoma
4134-I	Seminoma	
4332-I	Seminoma	
5220-I	Seminoma	
5225-I	Seminoma	
5423-I	Seminoma	
3522-I	combined tumor	Seminoma + Teratoma + Endodermal Sinus Tumor
3769-I	combined tumor	mainly Seminoma + very little Embryonal Carcinoma
4037-I	combined tumor	Seminoma + Teratoma + Endodermal Sinus Tumor + Embryonal Carcinoma
4217-I	combined tumor	Seminoma + Embryonal Carcinoma
5158-I	combined tumor	Teratocarcinoma + Seminoma

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

```
                                                                  M   R   A   P       4
acatctacgcagcgaaatcgagcctggccttgagggtccacaccgcgaggggaag ATG CGT GCG CCC     66

 I   P   E   P   K   P   G   D   L   I   E   I   F   R   P   F   Y   R      22
ATT CCA GAG CCT AAG CCT GGA GAC CTG ATT GAG ATT TTT CGC CCT TTC TAC AGA    120

 H   W   A   I   Y   V   G   D   G   Y   V   V   H   L   A   P   P   S      40
CAC TGG GCC ATC TAT GTT GGC GAT GGA TAT GTG GTT CAT CTG GCC CCT CCA AGT    174

E   V   A   G   A   G   A   A   S   V   M   S   A   L   T   D   K   A      58
GAG GTC GCA GGA GCT GGT GCA GCC AGT GTC ATG TCC GCC CTG ACT GAC AAG GCC    228

 I   V   K   K   E   L   L   Y   D   V   A   G   S   D   K   Y   Q   V      76
ATC GTG AAG AAG GAA TTG CTG TAT GAT GTG GCC GGG AGT GAC AAG TAC CAG GTC    282

 N   N   K   H   D   D   K   Y   S   P   L   P   C   S   K   I   I   Q      94
AAC AAC AAA CAT GAT GAC AAG TAC TCG CCG CTG CCC TGC AGC AAA ATC ATC CAG    336

 R   A   E   E   L   V   G   Q   E   V   L   Y   K   L   T   S   E   N     112
CGG GCG GAG GAG CTG GTG GGG CAG GAG GTG CTC TAC AAG CTG ACC AGT GAG AAC    390

 C   E   H   F   V   N   E   L   R   Y   G   V   A   R   S   D   Q   V     130
TGC GAG CAC TTT GTG AAT GAG CTG CGC TAT GGA GTC GCC CGC AGT GAC CAG GTC    444

 R   D   V   I   I   A   A   S   V   A   G   M   G   L   A   A   M   S     148
AGA GAT GTC ATC ATC GCT GCA AGC GTT GCA GGA ATG GGC TTG GCA GCC ATG AGC    498

 L   I   G   V   M   F   S   R   N   K   R   Q   K   Q                     162
CTT ATT GGA GTC ATG TTC TCA AGA AAC AAG CGA CAA AAG CAA  taactgaaaaagact  555

gtcctgtcagcgatgactttatacatcaaggggtcttgttttgctagagagtttggggtttggtttgtgga  627

tttcattgtgatttataataaggcttattttcacagaataaaataaagc                        676
```

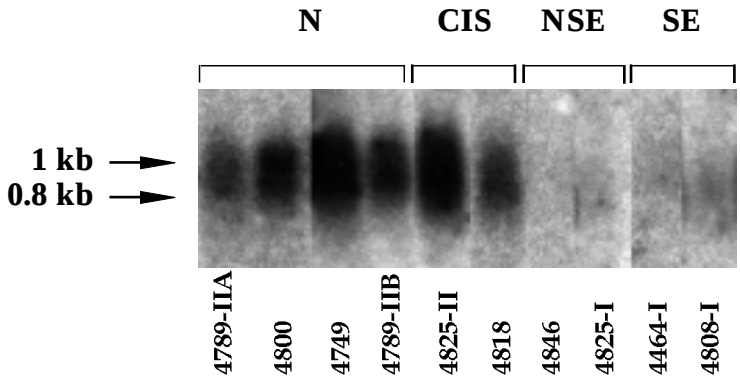
Hal-Inserm Author manuscript



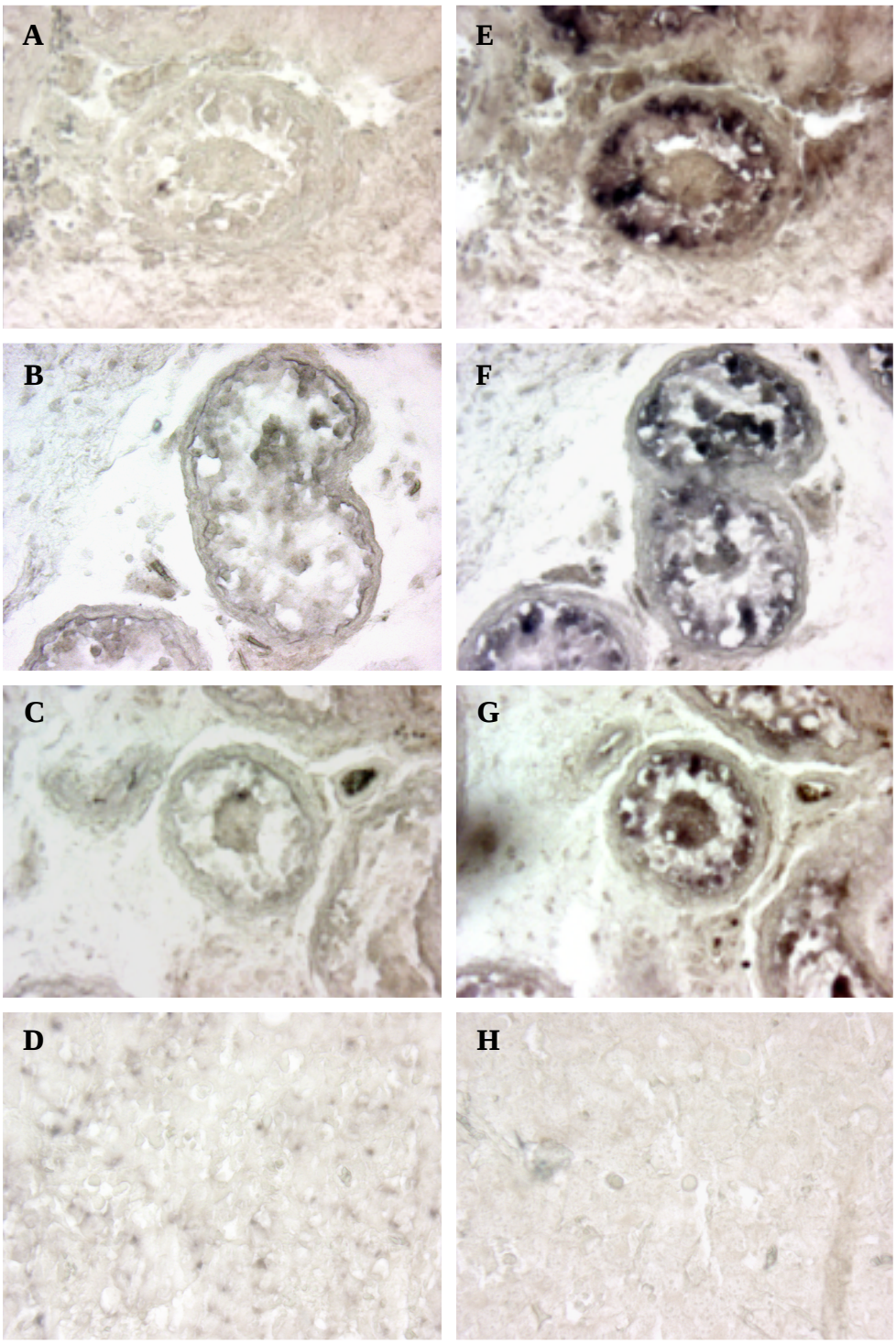
Hal-Inserm Author manuscript

Hal-Inserm Author manuscript

Hal-Inserm Author manuscript



Hal-Inserm Author manuscript



Hal-Inserm Author manuscript