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(54) Title: HOMOZYGOUS DELETION OF CHROMOSOME 8p23 IN HEPATOCELLULAR CARCINOMA

(57) Abstract: A method for detecting human hepatocellular carcinoma (HCC), wherein the method comprises detecting a homozygous deletion in human chromosome 8p23.

Homozygous Deletion of Chromosome 8p23 in Hepatocellular Carcinoma

The present invention relates to a method for detecting human hepatocellular carcinoma (HCC), wherein the method comprises detecting a homozygous deletion in human chromosome 8p23.

The identification of several major tumor suppressor genes (TSG) in the last decade has brought substantial gains in the fundamental understanding of two common human malignant tumors : breast and colorectal cancers. Notably, advances in molecular genetics lead to a better characterization of biological behaviors of tumors associated with specific TSG mutations. Ultimately, such progress would benefit patients at high risk of recurrence or metastasis and lead to the definition of appropriate treatments. However, such a propitious situation is not found in the field of primary liver cancer.

Hepatocellular carcinoma (HCC), the most frequent histological form of primary liver cancer, is one of the most prevalent human tumors with more than 400,000 new cases diagnosed each year worldwide. The natural history of HCC, generally diagnosed in an advanced form, is characterized by very poor survival rates. Several studies have recently demonstrated a significant increase in its incidence in the past twenty years in Japan, USA, and Europe. This increased incidence is thought to reflect the strong impact of some infectious or environmental factors on the pathogenesis of the tumor. The importance of chronic infection with hepatitis B and C viruses (HBV/HCV) in HCC has been well documented all over the world and, taken together, these viral infections are present in more than 80% of new primary liver cancer cases . In addition, at least in some geographical areas, chemical carcinogenic compounds (essentially alcohol and aflatoxin B1) represent major risk factors of primary liver cancer. The available data in Europe suggests a further important rise in HCC incidence. This, as well as the major role of hepatitis viruses in this cancer, put forward this tumor as a major health care problem.

Until recently, the genetic abnormalities occuring in HCC were poorly defined. Indeed like a majority of epithelial tumors, HCC is highly refractory to classical cytogenetic analysis. In addition, the lack of specific translocations and the complexity of the genomic rearrangements found in each solid tumor have been responsible for the slow progress in the genetic analysis of this particular type of neoplasia. Consequently, only a handful of tumor suppressor genes (TSG) or

protooncogenes potentially implicated in liver carcinogenesis have been carefully studied: CDKN2A (in 9p21 on human genome), H/K/N-RAS (11p15, 12p12, 1p13, respectively), PTEN (10q23) (Challen *et al.*, 1992, Biden *et al.*, 1997, Yao *et al.*, 1999), p53 (17p13), and the b-catenin gene (3p21).

The human cell has 23 pairs of chromosomes. Every cell has two copies of a gene. Each copy is located on a chromosome in a chromosome pair, called a gene pair. Alleles are alternative forms of a gene. When a cell has two different alleles that constitute the gene pair, it is considered to be heterozygous for that gene. In contrast, when a cell has identical alleles that constitute a gene pair, it is considered to be homozygous. An allelotype is a global profile of a chromosome(s) that identifies the alleles present for each gene analyzed on the chromosome(s). In this invention, a genome-wide scanning was done. In some cases, a heterozygous person can have one dominant normal allele that masks the effect of a second mutated, cancerous allele in the gene pair. As with the case of Rb (retinoblastoma), loss of the normal allele, and thus loss of heterozygosity (LOH), results in a cessation of the masking effect. This allows the cancerous trait to emerge, which makes it possible to look for a loss of heterozygosity in identifying and locating (mapping) potential tumor suppressor genes responsible for hepatocellular carcinoma.

LOH assays previously allowed to define regions that colocalize with already identified tumor suppressors, like p53 on 17p. Yet, it is remarkable that within the most prominent LOH regions, 8p, 4q, 13q, and 6q, liver TSG still remain to be identified (Nagai *et al.*, 1997)

A secondary allelotyping was conducted on chromosomal arm 8p to better define the regions of preferential heterozygosity loss, which might carry putative tumor suppressor genes. Three distinct minimal deleted areas were defined : a 13 cM region in the distal part of 8p21, a 9 cM area in the more proximal portion of 8p22 and a 5 cM area in 8p23 (Pineau *et al.*, 1999).

The present invention relates to the refinement of the genetic map and positional cloning of TSG located on chromosome 8p. To this end, the inventors used an approach different from the mapping using the LOH assay. The method according to the invention consists in a search for homozygous deletions (HD) on chromosome 8p.

According to one aspect, the invention relates to an isolated nucleic acid having a sequence selected from the group consisting of SEQ ID n° 37, SEQ ID n° 38, SEQ ID n° 39, SEQ ID n° 40, SEQ ID n° 41, SEQ ID n° 42, SEQ ID n° 43, SEQ ID n° 44, SEQ ID n° 45, SEQ ID n° 46, SEQ ID n° 47, SEQ ID n° 48, SEQ ID n° 49, SEQ ID n° 50, SEQ ID n° 51, SEQ ID n° 52 and SEQ ID n°53, and a homologous sequence thereof. Oligonucleotides comprising one of the above mentioned sequences are also within the scope of the invention provided that they allow for the detection of an homozygous deletion on chromosome 8p23, preferably within a 345 Kb region flanked by the 370L3SP6 and 315l17fg8D loci markers.

As used herein, The terms « isolated » and « purified » according to the invention refer to a level of purity that is achievable using current technology. The molecules of the invention do not need to be absolutely pure (i.e., contain absolutely no molecules of other cellular macromolecules), but should be sufficiently pure so that one of ordinary skill in the art would recognize that they are no longer present in the environment in which they were originally found (i.e., the cellular middle). Thus, a purified or isolated molecule according to the present invention is one that have been removed from at least one other macromolecule present in the natural environment in which it was found.

More preferably, the molecules of the invention are essentially purified and/or isolated, which means that the composition in which they are present is almost completely, or even absolutely, free of other macromolecules found in the environment in which the molecules of the invention are originally found. Isolation and purification thus does not occur by addition or removal of salts, solvents, or elements of the periodic table, but must include the removal of at least some macromolecules.

"A homologous nucleic acid sequence" is understood as meaning a sequence which hybridizes with the sequences to which it refers or to their complementary sequences under the usual conditions of stringency (Sambrook et al, 1989) so long as said homologous sequence shows at least 70 % of homology, preferably 90 % of homology with the above-defined sequences.

The homology can be determined, for example by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (Nucl. Acids Res. 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignement

method of Needleman and Wunsch (J. Mol. Biol. 48:443, 1970), as revised by Smith and Waterman (Adv. Appl. Math 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (Nucl. Acids Re. 14:6745, 1986) as described by Schwartz and Dayhoff, eds., (Atlas of Protein Sequence and Structure, National Biomedical Search Fundation, pp. 353-358, 1979) (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end.

As an illustrative embodiment, the stringent hybridization conditions used in order to specifically detect a polynucleotide according to the present invention are advantageously the following:

Prehybridization and hybridization are performed at 68°C in a mixture containing:

- 5X SSPE (1X SPE is 0.18 M NaCl, 10mM NaH2PO4.);
- 5X Denhardt's solution;
- 0.5% (w/v) sodium dodecyl sulfate (SDS); and
- 100 mg ml-1 salmon sperm DNA.

The washings are performed as follows:

a) Two washing at laboratory temperature for 10 min in the presence of 2X SSPE and 0.1% SDS;

b) One washing at 68°C for 15 min. in the presence of 1X SSPE and 0.1% SDS; and

c) One washing at 68°C for 15 min. in the presence of 0.1X SSPE and 0.1% SDS.

Nucleic acids as primers allowing for the detection of a homozygous deletion on chromosome 8p23, preferably within a 345 Kb region flanked by the 370L3SP6 and 315l17fg8D loci markers, belongs to the invention. These nucleic acids comprise at least 15 nucleotides, preferably at least 18 nucleotides, and preferably less than 40 nucleotides.

The invention also relates to nucleic acids as probes. Such nucleic acids comprise at least 15 nucleotides, preferably at least 20 nucleotides, still preferably at least 100 nucleotides, but are preferably shorter than 2000 nucleotides. A probe of

sequence SEQ ID n°53, or any probe contained in and/or showing homology to SEQ ID n° 53 is within the scope of the invention

In another aspect, the invention relates to the use of an isolated nucleic acid, according to the invention for in vitro detecting a homozygous deletion in human chromosome 8p23. In a preferred embodiment said homozygous deletion is detected within a 345 Kb region flanked by the 370L3SP6 and 315l17fg8D loci markers. Said isolated nucleic acid are advantageously used for in vitro detecting a human hepatocellular carcinoma.

The present invention also comprises a method for in vitro detecting human hepatocellular carcinoma (HCC) wherein the method comprises detecting a homozygous deletion in human chromosome 8p23. Preferably, the homozygous deletion is detected within a 345 Kb region flanked by the 370L3SP6 and 315l17fg8D loci markers.

According to a particular embodiment, the homozygous deletion is identified by hybridization techniques using an oligonucleotide probe specific for the deleted region. Suitable hybridization techniques are well known by the skilled in the art.

In another embodiment, said homozygous deletion is identified by PCR amplification and/or RDA using a suitable primer pair. Nucleic acids as primers allowing for the detection of a homozygous deletion on chromosome 8p23, preferably within a 345 Kb region flanked by the 370L3SP6 and 315l17fg8D loci markers, comprise at least 15 nucleotides, preferably at least 18 nucleotides, and preferably less than 40 nucleotides. In a particular embodiment, a primer pair is selected from the group consisting of SEQ ID n° 37 and SEQ ID n° 38, SEQ ID n° 39 and SEQ ID n° 40, SEQ ID n° 41 and SEQ ID n° 42, SEQ ID n° 43 and SEQ ID n° 44, SEQ ID n° 45 and SEQ ID n° 46, SEQ ID n° 47 and SEQ ID n° 48, SEQ ID n° 49 and SEQ ID n° 50, SEQ ID n° 51 and SEQ ID n° 52. Any primer based equivalent technique known by the skilled in the art is also suitable.

A kit for diagnosing human hepatocellular carcinoma comprising an isolated nucleic acid having a sequence selected from the group consisting of SEQ ID n° 37 to SEQ ID n° 53, and a homologous sequence thereof is within the scope of the invention.

Tumor suppressor genes often function as regulators of normal cellular processes. An alternative genetic mechanism to LOH that leads to cancer is homozygous deletion wherein both copies of the gene are deleted from the chromosome, The consequent loss of the normal protein would result in a loss of the regulator function of the TSG, allowing normal cellular events (such as cell division) to proceed unchecked. A deletion is the removal or the absence of a genetic sequence from the chromosome. Homozygous deletions occur when both genes in a gene pair have been deleted.

A collection of 110 tumor cell lines was obtained. More than half of them were from hepatobiliary origin. The remaining were established from some of the tumor types exhibiting high rates of LOH on chromosome 8p, *i.e.* breast, ovarian, head, and neck squamous cell carcinomas, as well as non-small- and small-cell lung cancers. In contrast to tumorous samples obtained *ex vivo,* tumor cell lines are advantageously not contaminated with normal cells and represent consequently a *biological material of choice for HD detection.*

ESTs, STRPs and STSs represent different types of unique genetic targets that lie throughout the chromosome. These targets can be assessed for the complete absence of the target, a homozygous deletion, or an actual sequencing of the target to determine mutations that have occurred. The complete loss of the sequence has the same global effect as a loss of heterozygosity in that the function of the normal wild type gene is lost. This invention concentrates on targets that lie in loci (locations) of chromosome 8p identified as experiencing deletions. Expressed Sequence Tags (ESTs) are small areas of known DNA sequence that lie in genes that are actively expressed in the cell. Simple Tandem Repeat Polymorphisms (STRPs) are small areas in the chromosome in which a sequence has been duplicated, wherein the repeats lie next to each other. Short Tagged Sequences (STSs) represent known areas of unique human DNA sequence.

Cell lines were screened by STS marker scanning, *i.e.* for the presence of amplification products (amplimers) for Expressed Sequence Tags (*ESTs*), Simple Tandem Repeat Polymorphism (STRP), and Short Tagged Sequences (*STS*) located every 250 kb in the critical loci of deletions. An absence of amplimer in such regions, hallmark of a HD, would be indicative of the presence of a candidate TSG.

The search for HD is achieved using standard PCR or substractive techniques as Representational Difference Analysis (RDA) (Lisitsyn *et al.*,

1993)*Representational Difference Analysis (RDA)* is a method in which one can amplify out sequences that are deleted in an abnormal (i.e. cancerous) genome. For example, to ask if there are sequences deleted from chromosome 8p HCC cell line, one would isolate the DNA from that cell line and isolate the DNA from a normal cell line of the same lineage (i.e. liver cells). DNA sequences that are in common between the two cell lines will hybridize. DNA sequences present in the normal DNA but are deleted in the abnormal DNA will have nothing to hybridize to. The hybridized sequences are subtracted out, leaving only the unbound sequences that are deleted in the abnormal DNA that are subsequently amplified for further identification.

[001] The search for HD in HCC on 8p as well as on other chromosomes has not been seriously undertaken so far. However, a few HD have been already found on chromosome 8p in 3 tumor types : prostate, pancreas, and squamous cell carcinoma (CC). Accordingly, different types of biological specimens were used, and different segments of chromosome 8p were found homozygously deleted (cf Table 1 below).

TABLE 1

Tissues	**Samples**	**Homozygous Deletion**			**Author**
		cytogenetics	loci involved	app. size	
Prostate	tumors	8p22	D8S1991-1992	750 kb	(Bova *et al.*, 1996)
Prostate	tumors	8p12	D8S87	<1400 kb	(Prasad *et al.*, 1998)
Prostate	cell-lines	8p12-21	D8S1769-2162	730-1320 kb	(Van Alewijk *et al.*, 1999)
Pancreas	cell-lines	8p22	D8S1991-1992	450 kb	(Levy *et al.*, 1999)
Squamous CC	tumor	8p23	D8S262-1806	?	(Ishwad *et al.*, 1999)
Squamous CC	cell-lines	8p23	D8S1788-1824	?	(Sun *et al.*, 1999)

Legend of Table 1 : CC, cell carcinoma ; kb, kilobases, app size, approximate size.

The 8p STS mapping in the minimal regions of loss in 8p21, 8p22 and 8p23 (10, 8 and 5 megabases, respectively) were selected on a chromosome 8 contig established by the University of Southampton (United-Kingdom,

ftp://cedar.genetics.soton.ac.uk/pub/chrom8/). This high-density contig is a quasi-exhaustive compendium of available markers (*EST, STRP,* and *STS*) mapping on chromosome 8. Some additional public markers were added to our study (as D8S1991, D8S1992). The average density of STS, on this contig is 200 kb. Such a tool associated to our large collection of tumor cell lines was appropriate to detect small HD in our 3 regions of interest.

The locations of these STS targets were identified (mapped) in three specific areas of chromosome 8 identified as having deletions associated with HCC. Chromosome nomenclature follows a specific pattern. The first number identifies the chromosome (8), the first letter identifies the arm of the chromosome (p) which could be p or q, and the next number identifies the specific position on the arm (i.e. 21, 22 or 23). The physical size of each area on the chromosome is given in megabases. Each megabase is 1,000,000 bases or nucleotides in the DNA. For example, 8p21 is 10^7 bases long. A contig is a contiguous map of these STS targets in chromosome 8, giving their location on the chromosome and distance from each other. The average space between each target or marker is only 200 kilobases or 2 X 10^5 bases, which is closely spaced and thus high-density. The closer these markers are to each other, the more accurately one can map the location of a deletion or sequence change on the chromosome. In addition, the smaller the sequence between markers, the easier it is to detect small deletions between those markers. Thus, the more markers that are known, the better.

False positive results (*i.e* absence of amplimeres) attributable to polymorphisms in primer sequence may be ruled out by the design of new primers on the same locus. Subsequently to HD detection, their sizes and borders are be determined by fluorescent in situ hybridization (FISH) on interphase nuclei using probes prepared from YAC, BAC or PAC containing the deleted amplimers. This experiment allowed to further reduce the size of the chromosome segment to study.

This invention identifies regions of chromosomes that sustain a homozygous deletion and that are associated with hepatocellular carcinoma. Initially techniques such as allelotyping and comparative genomic hybridization were used on HCC tumor samples to broadly identify the locations of LOH and HD down to the specific chromosome and to which arm of the chromosome the alteration maps. Among the areas identified using these techniques was chromosome 8, arm p (8p). Because of

the high frequency of LOH and its association with a variety of other cancers and tumors, the focus was on 8p to produce a finer, more detailed map of the deletions present. To this end, high-density polymorphic marker analysis and STS marker scanning were used to identify deletions in three regions of 8p, those being 8p21, 8p22, and 8p23. In the process, a very useful tool for future analysis of 8p is provided. A fine genetic map of several specific markers located in 8p that are closely spaced together have been identified.

This body of work represents the identification of one or more TSG involved in the development of HCC. As the invention stands, these specific deletions, to the detail presented here, can be used as genetic markers to diagnose the propensity of a patient for developing HCC. The addition made by the invention to the contig of 8p results in a very detailed map of several marker types. Such a map is very useful to others interested in doing different types of cytogenetic analysis on this region of chromosome 8.

MATERIALS AND METHODS

HUMAN CELL LINES AND DNA EXTRACTION

A total of 95 human cell lines included 58 hepatobiliary and 37 non hepatobiliary cell lines were extensively cultured to 30 million of cells. High molecular weight genomic DNAs were extracted and purified as described previously by sambrook et al. (Sambrook *et al.*, 1989). DNAs were quantified in an optic densitometer and homogeneously diluted to a concentration of 50 ng/ml.

PCR ANALYSIS

The 95 human cell lines were assayed for deletion by PCR with 43 primer pairs located in the MRL3 region between D8262 and D8S1825. The primers have been choosen to be distributed every 250 kilobases in this region. The sequences of the oligonucleotides and their distribution are freely publicly available on the electronic databases, particularly on the Internet World Wide Web at the following address:" http://cedar.genetics.soton.ac.uk/pub/chrom8/map.html". It contains microsatellite as well as STSs markers (Simple Tag Sequences) distributed as follows:

D8S262

D8S1824
D8S201
D8S7
D8S1042
D8S1140
D8S1806
D8S1099
D8S307
D8S1798
D8S518
D8S1963
D8S1788
D8S1781
WI-22787 (renamed STS302)
248WA9 (renamed STS315)
D8S1742
N54165 (renamed STS304)
A008R37 (renamed STS303
STSG43139 (renamed STS305)
AA011655 (renamed STS306)
D8S561
STSG30148 (renamed STS307)
STSG12852(renamed STS308)
STSG39654 (renamed STS309)
STSG2176 (renamed STS310)
A005M25 (renamed STS314)
STSG16046 (renamed STS311)
STSG42716 (renamed STS312)
D8S277
STSG10291(renamed STS313)
STSG3758 (also D8S141)
D8S1511
D8S1819
D8S1706

D8S1935
D8S439
D8S252
D8S516
D8S503
D8S1469
D8S1825
D8S349

PCRs were performed in a final 25 ml reaction volume including 50 ng of genomic DNA, 20 pmol of each primer, 1.25 mM dNTPs, 1 unit of Taq polymerase and 1X PCR buffer (10mM Tris (pH8.9), 0.1% Tween 20, 1.5 mM MgCl2, 50 mM KCl). An initial denaturation step was performed during 3.5 min at 94°C. Amplification were carried out during 35 cycles of denaturation (94°C for 45 sec) and annealing (48 to 58 °C for 1min) and an elongation (72°C for 1 min). At the end of the last cycle, samples were incubated for 4 min for complete elongation. PCRs reaction were loaded on an 2% agarose gel containing 0.5 mg/ml of ethidium bromide.

The presence or absence of an amplified products were estimated on an UV band luminometer. Each negative sample was reamplified in the same condition. If the second amplification confirmed the absence of PCR product, nested primers of the corresponding fragment are designed and tested on the negative DNAs.

D8S503bp: 5'GTTCAAATTGTCTCTAATGG 3' [SEQ ID NO: 1]
D8S503bj: 5'CTTACACATCGCTCAGAAAC 3' [SEQ ID NO: 2]
D8S262bs: 5'CTTGTATGTATATAAACGCC 3' [SEQ ID NO: 3]
D8S262bj: 5'GCTGATCATGGTACCACATG 3' [SEQ ID NO: 4]
D8S1824bp: 5'CTTCCAGCGTTTATTGCATC 3' [SEQ ID NO: 5]
D8S1824bj: 5'TTGCCAGTCAGTATGTCAAG 3' [SEQ ID NO: 6]
D8S1788bp: 5'CATTAAATTTGTAGCTACAG 3' [SEQ ID NO: 7]
D8S1788bj: 5'TTTTCACTATGCGTGCATAC 3' [SEQ ID NO: 8]
STS303be: 5'GATCTAGATGAAGAAATGG 3' [SEQ ID NO: 9]
STS303bj: 5'CAAATACTTAGAATCATCC 3' [SEQ ID NO: 10]
D8S1781Bp: 5'ACAGGGGTGACACTTCACAG 3' [SEQ ID NO: 11]
D8S1781Bj: 5'ATGTTCACATCTCCTGAAGC 3' [SEQ ID NO: 12]

STS303CE: 5'AAGAAGTGCAGAAGGAAG 3' [SEQ ID NO: 13]
STS303dj: 5'CTAGATGAAGAAATGGGG 3' [SEQ ID NO: 14]

Only the Li7A hepatobiliary cell line presented a deleted region included the D8S262, D8S1824, D8S1781, D8S1788 markers. These four markers, as well as D8S201, D8S1798, D8S 1806 and D8S264, were tested on 22 additional non hepatobiliary cell lines. No deletion have been observed.

In reference to the 8p map disposible on "http://cedar.genetics.soton.ac.uk/pub/chrom8/map.html" World Web Site, the D8S262, D8S1824, D8S1781, D8S1788 markers did not appear to be connected. The sequences of the four amplified products have been compared to the htgs unfinished High Throughput Genomic Sequences using the" http://www.ncbi.nlm.nih.gov/blast/blast.cgi" Internet World Wide Web site. Only the D8S262 marker showed a 100% homology with the sequence of an human BAC clone named 188e04 located on 8p23, sequenced by the genome Sequencing Centre at the Institute of Molecular Biotechnology, Iena, Germany. The freely publicly available complete sequence of the BAC clone 188e04 allowed us to designe primers in an unique region located near the ends of the BAC to confirmed that the proximal region of D8S262 is lost and if the other end, distanced of 170028 bp, extends the deleted region in the Li7A cell line. The sequences of the primers are:

188e4-Nf: 5'GGAGTGAGTCCAGAGATTCT 3' [SEQ ID NO: 15]
188e4-Nr: 5' TCACACAGGATTTCAACAGA 3' [SEQ ID NO: 16]
188e4-Cf: 5'TCACAGGTAAAGGCTGAAG 3' [SEQ ID NO: 17]
188e4-Cr: 5'AGAAGGGCAATCTGTGAGTA 3' [SEQ ID NO: 18]

Both ends of the 188e04 BAC clone are lost in the Li7A cell line extending the deleted region of 168511 bp. Comparison of the 188e04 sequence with the Data Bank of the htgs unfinished High Throughput Genomic Sequences on the" http://www.ncbi.nlm.nih.gov/blast/blast.cgi" Web site revealed that the distal region to D8S262 is highly homologous to an other BAC clone named 315_I_17 sequenced by the Whitehead Institute/MIT Center for Genome Research, USA . The freely publicly 'working draft' sequence of this BAC is constituted of 9 contigs.

1 9370: contig of 9370 bp in length
9371 12536: contig of 3166 bp in length

12537	22556: contig of 10020 bp in length
22557	33359: contig of 10803 bp in length
33360	49987: contig of 16628 bp in length
49988	71597: contig of 21610 bp in length
71598	102069: contig of 30472 bp in length
102070	169862: contig of 67793 bp in length
169863	174219: contig of 4357 bp in length.

The true order of the contigs is not known and their order in this sequence record is arbitrary. the exact sizes of the gaps between the contigs are unknown.

The contig: 49988; 71597 of 21610 bp in length corresponded to the last fragment containing 188e04 sequences . A primer pair located at the ends of the 315_I17 BAC clone were designed:

315I17aS	gagggcactttctttgtatg	[SEQ ID NO: 19]
315I17aAS	cccaaggtatatttcctcct	[SEQ ID NO: 20]

The corresponding amplified sequence is not present in the Li7A cell line, extending the deleted region of 67788 bp.

In parallel, a BAC clone named 2003M15, has been freely provided by the genome Sequencing Centre at the Institute of Molecular Biotechnology, Iena, Germany, as overlaping the 188e04 clone in the orientation than the 315-I17 clone.

A recent publication by Sun *and al.* (Sun *et al.*, 1999) concerning the identical region, determined a panel of new STS markers defined by their BAC contig. These markers: 341B24-T7, 341B24-SP6, 309K3-T7, 309K3-SP6, 370L3-T7, 370L3-SP6, 563O9-T7, 459J20-SP6, 389E23-T7, 389E23-SP6, 254M4-T7, 254M4-SP6, 236F7-T7, 549J13-T7 have been tested on the Li7A cell line. All the markers located upstream D8S1824 are retained in Li7A restricted the telomeric boundary closed to D8S1824.

In the centromeric region, all the markers located downstream D8S262 and contained in the 188e04 BAC clone are lost, confirming previous data obtained by PCR analysis. The centromeric boundary of the deleted region in Li7A has been narrowed down using new primers generated from the sequences of the two contigs

33360; 49987 of 16628 bp in length and the 102070; 169862: of 67793 bp in length from the 315_I17 BAC clone, contigs that did not overlap with the 188e04 BAC clone. The generated primer pair sequences are:

315I17fg5f	ATTGCAGAGAGTAAGGCAAA	[SEQ ID NO: 21]
315I17fg5r	GCTGAAAGCTCTAAACAGGA	[SEQ ID NO: 22]
315I17fg8Nf	CGGTTTGCCAGTATTTTAT	[SEQ ID NO: 23]
315I17fg8Nr	TACCAGAGGTACAAGGAGGA	[SEQ ID NO: 24]
315I17fg8Cf	TGTAGTCCAGGTCTGAGCTT	[SEQ ID NO: 25]
315I17fg8Cr	TAAACGTAGTCCTTGTGGCT	[SEQ ID NO: 26]
315I17fg8Af	AAGGAAAAAGTGAGAGGACC	[SEQ ID NO: 27]
315I17fg8Ar	CCTGTAGTCCCAGCTACTTG	[SEQ ID NO: 28]
315I17fg8Bf	AACTATTAACGGCTGTTTGC	[SEQ ID NO: 29]
315I17fg8Br	TCTATTTCTGGGGCATCTTA	[SEQ ID NO: 30]
315I17fg8Df	ATACAGCAATGTGCTCTCCT	[SEQ ID NO: 31]
315I17fg8Dr	AGTGGGGGTAGGAGTAATGT	[SEQ ID NO: 32]
315I17fg8Ef	CTGTGGTTGGAAGTCAGATT	[SEQ ID NO: 33]
315I17fg8Er	GAGCTGGTTACACAAAGAGG	[SEQ ID NO: 34]
315I17fg8Ff	ACATTCTCTCCAAACAGTGG	[SEQ ID NO: 35]
315I17fg8Fr	CACCCTACCTCATCCAATTA	[SEQ ID NO: 36]

The amplimer obtained with the sequence using the 315I17fg8B sets corresponds to the most proximal locus involved in the homozygous deletion in Li7A cell line. The total size of the homozygously deleted region in Li7A flanked by the 370L3SP6 and 315I17fg8D loci markers represents a maximum of 345 Kb.

The inventors have identified the location of deletions containing the tumor suppressor gene in region 8p23. Several probes (amplifiers) that span the deletions containing the tumor suppressor gene, primers for amplifying the deletion containing the tumor suppressor gene and primers for amplifying regions of the chromosome that abut the deletion have also been designed.

Nuclear acid probe, 544 pb

:Xd71CX12

ATgACAGATACGATTTAATCGAcTCCACTaTAaGGATTGCCTcGAAGGCAAGaATT
CGGGCaCGAGGGgATTCTTAGAgATCAGgTGGcaCGAAAGCTCcATCATAtGGcTA

AcTGGGCATAaCCCTccccCCCTCAGTATCAGAGCAAGaATTGGCTaCGAcTTCC
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ATGCTGTGTACGGAGGAGCATTTGCAGTTACAACACTTTGTAGCCATGCAGGAT
GGGGCAATTAATCCAGAACCATTATTTaataaaAAGATGATTTTTTAAATGTG
[SEQ ID NO: 53]

Primers allowing of the detection of deletions (double deletions)

D8S262BS: 5'CTTGTATGTATATAAACGCC 3' [SEQ ID NO: 37]
D8S262BJ: 5'GCTGATCATGGTACCACATG 3' [SEQ ID NO: 38]
D8S1824BP: 5'CTTCCAGCGTTTATTGCATC 3' [SEQ ID NO: 39]
D8S1824BJ: 5'TTGCCAGTCAGTATGTCAAG 3' [SEQ ID NO: 40]
D8S1788BP: 5'CATTAAATTTGTAGCTACAG 3' [SEQ ID NO: 41]
D8SI788BJ: 5'TTTTCACTATGCGTGCATAC T [SEQ ID NO: 42]
D8S1781BP: 5'ACAGGGGTGACACTTCACAG 3' [SEQ ID NO: 43]
D8S1781BJ: 5'ATGTTCACATCTCCTGAAGC 3' [SEQ ID NO: 44]
188e4-Nf: 5'GGAGTGAGTCCAGAGATTCT 3' [SEQ ID NO: 45]
188e4-Nr: 5'TCACACAGGATTTCAACAGA 3' [SEQ ID NO: 46]
188e4-Cf: 5'TCACAGGTAAAGGCTGAAG 3' [SEQ ID NO: 47]
188e4-Cr: 5'AGAAGGGCAATCTGTGAGTA 3' [SEQ ID NO: 48]

Amplimers with the breakpoint proximal (have 500 Pb near) double deletions (primers that abut the deletion) :
315I17fg8Df ATACAGCAATGTGCTCTCCT [SEQ ID NO: 49]
315117fg8Dr AGTGGGGGTAGGAGTAATGT [SEQ ID NO: 50]

Amplimers with the breakpoint distal :
370L3Sp6f GAGGATGTGACAGTATTGGATATCA [SEQ ID NO: 51]
370L3Sp6r GCCTTCCTATGAGCTGCACGG [SEQ ID NO: 52]

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WHAT IT CLAIMED IS:

1. Isolated nucleic acid having a sequence selected from the group consisting of SEQ ID n° 37, SEQ ID n° 38, SEQ ID n° 39, SEQ ID n° 40, SEQ ID n° 41, SEQ ID n° 42, SEQ ID n° 43, SEQ ID n° 44, SEQ ID n° 45, SEQ ID n° 46, SEQ ID n° 47, SEQ ID n° 48, SEQ ID n° 49, SEQ ID n° 50, SEQ ID n° 51, SEQ ID n° 52 and SEQ ID n°53, and a homologous sequence thereof

2. Use of an isolated nucleic acid, according to claim 1, for in vitro detecting a homozygous deletion in human chromosome 8p23.

3. Use, according to claim 2, wherein said homozygous deletion is detected within a 345 Kb region flanked by the 370L3SP6 and 315I17fg8D loci markers.

4. Use, according to any of claims 2 or 3, for in vitro detecting a human hepatocellular carcinoma.

5. A method for in vitro detecting human hepatocellular carcinoma (HCC), wherein the method comprises detecting a homozygous deletion in human chromosome 8p23.

6. A method, according to claim 5, wherein said homozygous deletion is detected within a 345 Kb region flanked by the 370L3SP6 and 315I17fg8D loci markers.

7. A method, according to claim 5 or 6, wherein said homozygous deletion is identified by hybridization techniques using an oligonucleotide probe specific for the deleted region.

8. A method, according to claim 7, wherein said probe has the sequence SEQ ID n°53, or any sequence contained in and/or showing homology to SEQ ID n° 53

9. A method, according to claim 5 or 6, wherein said homozygous deletion is identified by PCR amplification and/or RDA using a primer pair selected from the group consisting of SEQ ID n° 37 and SEQ ID n° 38, SEQ ID n° 39 and SEQ ID n° 40, SEQ ID n° 41 and SEQ ID n° 42, SEQ ID n° 43 and SEQ ID n° 44, SEQ ID n° 45 and SEQ ID n° 46, SEQ ID n° 47 and SEQ ID n° 48, SEQ ID n° 49 and SEQ ID n° 50, SEQ ID n° 51 and SEQ ID n° 52.

10. A kit for diagnosing human hepatocellular carcinoma comprising an isolated nucleic acid having a sequence selected having a sequence selected from the group consisting of SEQ ID n° 37, SEQ ID n° 38, SEQ ID n° 39, SEQ ID n° 40, SEQ ID n° 41, SEQ ID n° 42, SEQ ID n° 43, SEQ ID n° 44, SEQ ID n° 45, SEQ ID n° 46, SEQ ID n° 47, SEQ ID n° 48, SEQ ID n° 49, SEQ ID n° 50, SEQ ID n° 51, SEQ ID n° 52 and SEQ ID n°53, and a homologous sequence thereof.

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INSERM

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