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ONCOGENOMICS

Identification and characterization of CRG-L2, a new marker for liver tumor development

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Liver cancer is very common worldwide and the rates of hepatocellular carcinoma (HCC) have increased by over 70% in the last 2 decades in the US. Late diagnosis, because of the lack of clinical symptoms, and decreased hepatic function, because of underlying hepatic disease, lead to the extremely high mortality rates associated with HCC. Clearly, the identification of markers that are expressed early in the development of HCC and that are easily detected in high-risk patients would aid in early diagnosis and increased survival. We present the cloning and characterization of a novel gene, *CRG-L2* (Cancer related gene-Liver 2), which displays high expression in murine and human hepatocellular carcinomas. Using *in situ* hybridization, we show that *CRG-L2* mRNA levels are increased early during the development of liver tumors in C3H/HeJ mice, and that in normal tissues *CRG-L2* mRNA is restricted to the murine testis and human placenta. Its restricted expression in normal tissues and unique early upregulation during tumor development make *CRG-L2* an excellent candidate as a new clinical marker of HCC.
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Introduction

Primary liver cancer is the fifth most common cancer worldwide with approximately half a million cases reported in 1990. The fatality ratio (mortality/incidence) is approximately 1, indicating that the majority of patients with liver cancer live less than a year (Bosch *et al.*, 1999). Compared to breast and prostate cancers in the US, which have fatality rates of 0.2, the fatality rate of liver cancer is extremely high (American Cancer Society, 2002). The lower fatality rate of breast and prostate cancers may be because of the screening technologies (e.g. mammography and prostate-specific antigen screening) that allow the identification of breast and prostate carcinomas at early stages of development when they are easier to treat effectively. Ultrasonography, CT scans, and serological markers are being used to screen high-risk patients with hepatitis or cirrhosis for hepatocellular carcinomas (HCC); however, the inability to detect small lesions is a weakness in all of these techniques. For example, α-fetoprotein (AFP) is used as a serum marker for HCC but only 4.5–22% of patients with HCC have serum AFP values that exceed the diagnostic cutoff of 400 ng/ml and very few patients with small lesions have AFP values above this level (Song *et al.*, 2002). Since the incidence of HCC is on the rise in the US and worldwide (El-Serag and Mason, 1999), it is necessary to establish a more consistent and sensitive screening process. Therefore, additional markers for HCC must be identified. Some of the qualities of an effective HCC marker are (1) significant increased or decreased expression in comparison with normal liver, (2) deregulation in early stages of hepatocarcinogenesis, (3) lack of response to inflammation and proliferation caused by noncancerous states such as cirrhosis or hepatitis, (4) limited expression in normal tissues, and (5) the ability to be detected in bodily fluids, such as blood or urine.

To identify novel markers of HCC, we started with a mouse model because of the many disadvantages of using human tissue. For example, in comparing gene expression between normal tissues and neoplasias from humans, individual genetic variation must always be considered. By using inbred mouse strains, sample heterogeneity is avoided since each mouse has an identical genetic background. In addition, human HCC is often present in a background of cirrhosis or hepatitis, which must be considered when comparing expression patterns. Finally, a fundamental objective when studying tumorigenesis is to determine if the deregulated genes are involved in the initiation, promotion, or progression of a tumor. It is difficult to obtain early HCC lesions from patients. However, temporal studies can easily be conducted in a mouse model. In a previous study, we demonstrated that diethylnitrosamine (DEN)-treated C3 H/HeJ mice develop liver tumors that provide a representative model for human HCC (Graveel *et al.*, 2001). For example, we used oligonucleotide microarrays to identify a set of genes that are upregulated in mouse liver tumors and then showed that these genes are also upregulated in human liver tumors. We also cloned a novel gene that is

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upregulated in the mouse liver tumors and then showed that the human homolog of this gene displays increased expression in human liver tumors (Graveel *et al*., 2001). Owing to the similarity between gene expression in the livers of DEN-treated mice and human HCC, we have now cloned and characterized *CRG-L2* (Cancer related gene-Liver 2), a novel gene that corresponds to an mRNA fragment obtained using representational difference analysis to compare normal liver and liver tumors obtained from DEN-treated C3H/HeJ mice. We show that *CRG-L2* is upregulated in both mouse and human HCC, and that it possesses characteristics that suggest that it may be a potential new clinical marker for HCC and other cancers.

Results

Cloning of CRG-L2 using rapid amplification of cDNA ends (RACE)

By representational difference analysis, a 282 bp fragment of an uncharacterized mRNA was isolated (Graveel *et al*., 2001). Using RT–PCR analysis with primers located in the RDA fragment, this mRNA showed elevated expression in mouse liver tumors as compared to quiescent, regenerating, or newborn livers (Figure 1a). The low level of expression in the regenerating livers suggested the possibility that the increased expression was tumor specific and would not occur in nontumorigenic proliferative states of human liver, such as cirrhosis or hepatitis. Since this pattern of expression correlates with one of the characteristics for a good HCC marker (i.e. significant difference in expression in normal vs tumor tissue), the complete cDNA was obtained via RACE. Products from both 5′ and 3′ RACE were subcloned and sequenced. Sequencing the 3′ RACE products revealed three fragments that were identical at their 5′ ends because of the fixed location of the gene-specific primer. However, these fragments differed at their 3′ ends, with the longer fragments containing, but extending past, the sequence of the shorter fragments. Each fragment contained a polyA tail at its 3′ end, indicating that there are multiple polyadenylation sites. The 5′ RACE products were all identical. By conceptually combining the 5′ and 3′ RACE products, three mRNAs were identified that contained alternative 3′UTRs (Figure 1b). The putative start codon is at nucleotide 25 and the putative stop codon at nucleotide 1344. Since it was known that this mRNA was upregulated in murine liver tumors (yet the function was unknown), this novel mRNA was named Cancer related gene-Liver 2 (*CRG-L2*).

To confirm the presence of all three of the murine *CRG-L2* mRNAs and to determine which mRNA is predominantly expressed, a Northern blot hybridization

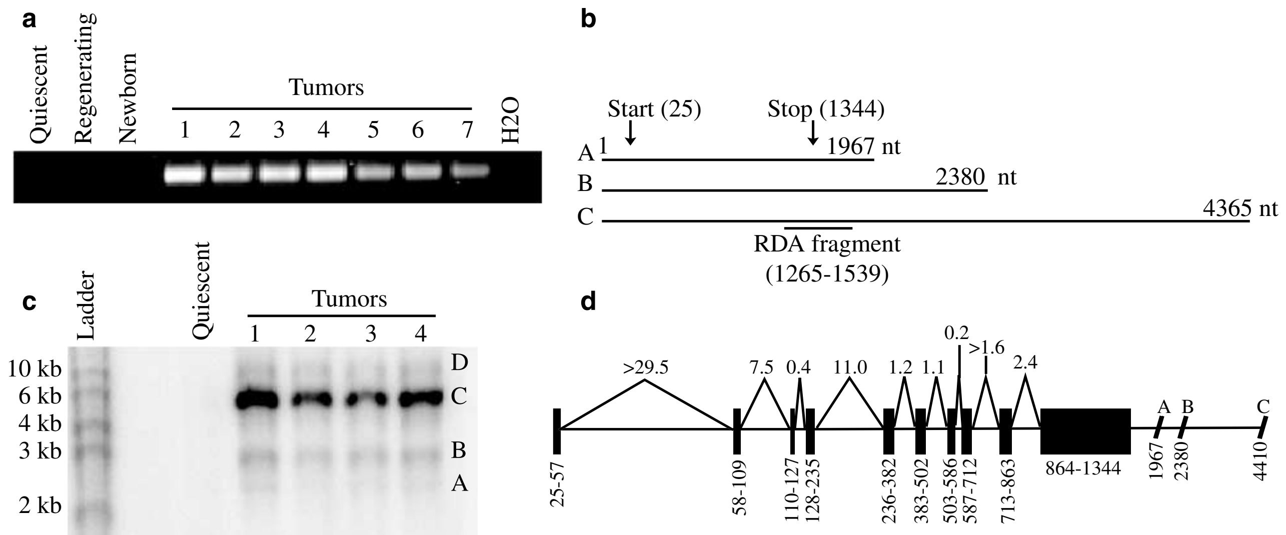

Figure 1 Cloning of CRG-L2: (**a**) RT–PCR analysis of *CRG-L2* in mouse liver tissues. Since the C3H/HeJ mice used in these studies are inbred, all untreated mice are genetically identical. Accordingly, we have never observed any differences in CRG-L2 expression in comparison with individual normal mice. Therefore, quiescent, regenerating, and newborn RNA samples were prepared from several mice and then pooled so that the same pooled RNA samples could be used in multiple experiments. Quiescent and regenerating samples are a combination of four livers and newborn samples are a combination of eight livers. However, it is known that tumors display heterogeneous genetic and molecular profiles. Therefore, to take into consideration these possible differences, the tumor samples used in our experiments are from individual mice. (**b**) mRNA structure of *CRG-L2*. Alignment of the 5′ and 3′ RACE products suggest that *CRG-L2* mRNA can contain one of the three alternative 3′UTRs. (**c**) Northern blot hybridization of *CRG-L2* in quiescent liver and four individual liver tumors. Four bands were detected at 2.4, 3.0, 5.5, and 10 kb. The three smaller mRNAs correspond to clones A, B, and C. A fourth band, D, was not cloned probably owing to inefficient PCR through a long 3′ UTR. (**d**) The *CRG-L2* open reading frame was aligned to mouse chromosome 9 (31 cM). Exons are represented by black boxes. The distance between some of the exons is estimated since there are gaps between the contigs in the genome and these gaps are represented by a > sign. *CRG-L2* is localized within chromosome 15q21.2 of the human genome and a similar intron/exon structure is suggested by comparing the mouse cDNA to the human genome

was performed using mRNA from quiescent livers and four individual liver tumors. A 1 kb fragment of the *CRG-L2* open reading frame was used as a probe and four mRNAs were observed (Figure 1c). The 2.4, 3.0, and 5.5 kb mRNAs (designated as A, B, and C, respectively, in Figure 1c) correspond to the 1967, 2380, and 4365 bp cloned cDNAs. The size of the observed mRNAs was longer than the RACE cDNA products owing to the polyA tails. A fourth mRNA (designated as D) of approximately 10 kb was faintly detected but was not cloned via RACE presumably because of its length. As expected, based on previous RT–PCR results, none of the mRNAs were observed in the quiescent livers. The 5.5 kb mRNA was the predominant form in the liver tumors and thus the sequence of the 4365 nt nucleotide mRNA has been deposited in Genbank as *CRG-L2* (AF548022).

To determine the structure of the *CRG-L2* gene, the sequence of the mRNA was aligned to mouse chromosome 9 (31 cM) using the Jackson Laboratory and Ensembl Mouse Genome browsers (Figure 1d). The *CRG-L2* gene is comprised of 10 exons and nine introns that cover a minimum of 59 kb. An exact measure of the *CRG-L2* gene is not yet possible because there are gaps between the contigs that contain the introns between exons 1 and 2 and exons 8 and 9. *CRG-L2* is localized within chromosome 15q21.2 of the human genome and a similar intron/exon structure is suggested by comparing the mouse cDNA to the human genome.

The putative amino-acid sequence of the predicted 47.5 kDa CRG-L2 protein was analysed by the SMART analysis program (Figure 2a), and was found to contain two collagen domains near the amino terminus (amino acids 29–88 and 89–149) and a large olfactomedin domain within the C terminus (amino acids 189–433). Hydrophobicity analysis of the putative CRG-L2 protein revealed hydrophobic sequences within the first 30 amino acids of the amino terminus, which represent a leader sequence, suggesting that CRG-L2 may be secreted (Figure 2b). A serine was also present at amino acid 21, which may be a potential cleavage site of the leader sequence. Regions of high hydrophobicity were also present in the carboxy-terminal region, which may represent potential transmembrane domains. The localization of CRG-L2 will be analysed in future experiments with immunofluorescence and cell fractionation studies.

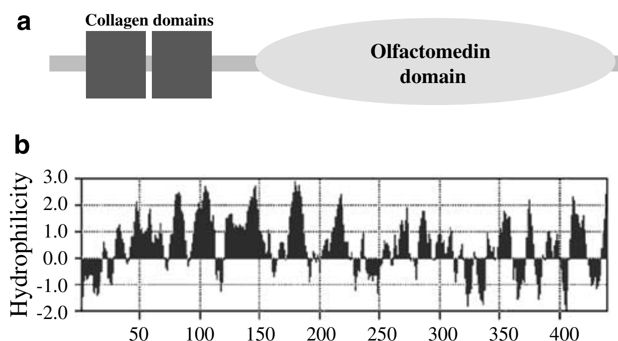

Figure 2 CRG-L2 protein analysis: (**a**) CRG-L2 cDNA encodes a putative protein of 48 kDa containing two collagen domains and an olfactomedin domain. (**b**) Hydrophobicity analysis of the putative CRG-L2 protein with the Kyle–Doolittle algorithm. Positive values represent hydrophilic regions and negative values represent hydrophobic regions

CRG-L2 *mRNA is upregulated in human hepatocellular carcinomas*

As noted above, regions of human chromosome 15 are highly similar to mouse *CRG-L2*. Based on this similarity, primers were designed to detect human *CRG-L2* mRNA. Using these human primers, the level of expression of *CRG-L2* was measured in multiple human hepatocellular carcinomas and in normal livers. A combined method of RT–PCR and Southern blot hybridization was used to measure the levels of human *CRG-L2*. *CRG-L2* mRNA was amplified by RT–PCR for 25 cycles and the PCR products were transferred to a nylon membrane that was probed with a fragment of the murine CRG-L2 open reading frame (188–1243). As shown in Figure 3, *CRG-L2* mRNA is essentially undetectable in the normal liver samples but can be detected in all five HCC samples (middle panel). Extremely high expression is seen in HCC-3, as seen by the shorter exposure of the film (top panel). Accurate quantitation of the starting mRNA samples was verified by the analysis of *GAPDH* mRNA.

CRG-L2 *is upregulated early in liver tumorigenesis*

The increased expression of *CRG-L2* in the mouse and human liver tumors suggested that further characterization as to its potential usefulness as a clinical marker was warranted. A very important characteristic of a clinical marker for HCC would be early expression during liver tumor development. As it is difficult to obtain samples corresponding to early states of liver tumors from human cancer patients, we investigated the timing of expression of *CRG-L2* using the DEN-treated mouse model. After a single administration of DEN to 12-day-old mice, basophilic foci are visible by histological staining at 12 weeks of age. Sequential develop-

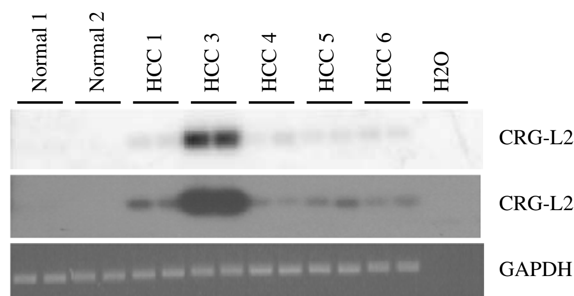

Figure 3 *CRG-L2* expression is increased in human HCC. Top panel is a phosphoimage of the RT–PCR results measuring *CRG-L2* mRNA, middle panel is a longer autoradiographic exposure. Equal loading was confirmed by the analysis of *GAPDH* mRNA. All HCC were classified as moderately differentiated

ment of hyperplastic nodules, hepatocellular adenomas, and hepatocellular carcinomas is observed between 12 and 32 weeks of age in male mice (Moore *et al*., 1981; Vesselinovitch *et al*., 1985). Therefore, we analyzed the DEN-treated mice at 20 and 32 weeks of age. At 20 weeks of age, numerous preneoplastic lesions were visible throughout the liver and by 32 weeks the foci had progressed into hepatocellular adenomas/carcinomas (Hanigan *et al*., 1988). Paraformaldehyde fixed sections from 20- and 32-week livers were probed with either an antisense (to detect *CRG-L2* mRNA) or sense (negative control) *CRG-L2* probe.

We began by analysis of the 32-week tumors because our RT–PCR results clearly showed that *CRG-L2* is upregulated at this stage. Although we expected to detect *CRG-L2* mRNA in the 32-week tumors, *in situ* hybridization can provide additional information that cannot be obtained by RT–PCR analysis. For example, tumor-specific genes may demonstrate a constant level of expression throughout a tumor or the expression can be localized to specific cell types or spatial locations (e.g. the periphery of the tumor). Using *in situ* hybridization, we observed that *CRG-L2* mRNA was upregulated in hepatocytes throughout the entire tumor (Figure 4a). We note that *CRG-L2* was detected in only 69% (311/453) of the tumors examined using *in situ* hybridization but was detected in all seven tumors examined by RT–PCR (Figure 1a). This could be because of the fact that only seven tumors were analysed in Figure la or because RT–PCR is more sensitive than *in situ* hybridization.

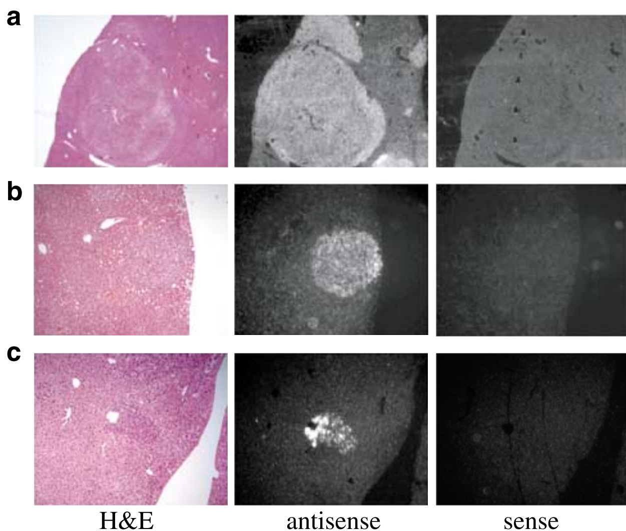

Figure 4 *CRG-L2* is upregulated early in liver tumorigenesis. *CRG-L2* expression was analysed by *in situ* hybridization in (**a**) 32 and (**b**) 20-week-old DEN-treated C3H/HeJ mice. *AFP* expression was analysed by *in situ* hybridization in (**c**) 20-week-old DEN-treated C3H/HeJ mice. Paraffin sections were hybridized to an [α^{35}S]UTP-labeled antisense or sense riboprobe derived from a fragment of the CRG-L2 open reading frame. Slides were dipped in emulsion, exposed for 14 days, and viewed at 2.5 × (**a**) or 10 × (**b,c**) under dark-field microscopy. H&E, antisense, and sense slides are serial sections

To determine if *CRG-L2* is upregulated at early stages of hepatocarcinogenesis, the expression of *CRG-L2* was examined in the preneoplastic foci using *in situ* hybridization. Interestingly, we found that *CRG-L2* mRNA can be detected in preneoplastic foci (Figure 4b). The pattern of *CRG-L2* expression appears to be consistent throughout the focus with no localization within any individual region. We found that *CRG-L2* is highly upregulated in 55% of the foci (220/403), but that there is no obvious histological differences in those foci that do or do not express *CRG-L2*: for example, *CRG-L2* is upregulated in both basophilic and eosinophilic foci and in foci with extensive fat or collagen deposits. *AFP* was found to be upregulated in 30% of preneoplastic foci (92/304), although the expression pattern was often restricted to various regions of the focus and not as uniformly distributed as *CRG-L2* (Figure 4c). In other studies, *AFP* has been shown to be expressed in only 23% of 28-week-old DEN-treated B6C3F1 mice (Koen *et al*., 1983) and 24% of human HCC by immunohistochemistry (Borscheri *et al*., 2001). In comparison with *AFP* in these studies, *CRG-L2* may be a more sensitive marker for the detection of early HCC.

CRG-L2 *displays restricted expression in normal tissues*

A characteristic of a good clinical marker for HCC is tumor-specific expression: that is, low expression in all normal tissues and not just in the tissue from which the tumor is derived. Although *CRG-L2* mRNA was not detected in normal mouse liver, it was possible that the mRNA was expressed in other normal tissues. The expression of *CRG-L2* was examined in mouse and human tissues using a multiple tissue cDNA panel. As performing high numbers of PCR cycles can sometimes obscure differential expression, aliquots of the PCR products were taken out after various cycles (30–34). We found that *CRG-L2* is primarily expressed in the mouse testis with moderate expression in skeletal muscle (Figure 5a). In human tissues, *CRG-L2* was expressed

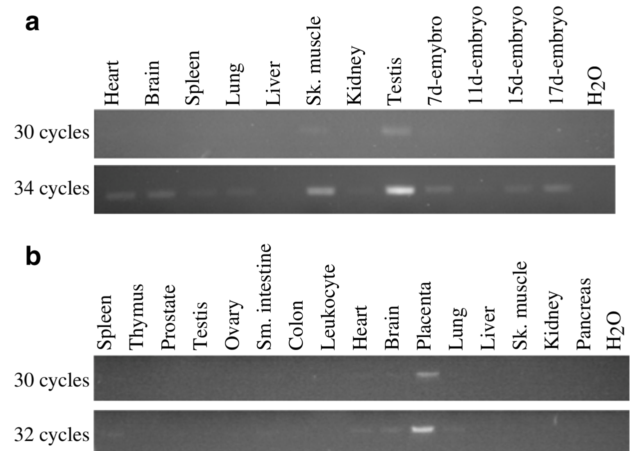

Figure 5 *CRG-L2* expression is restricted in normal tissues. *CRG-L2* mRNA was amplified in multiple mouse (**a**) and human tissues (**b**) using multiple tissue cDNA panels. Aliquots of the PCR products were taken out at the indicated cycles

primarily in the placenta (Figure 5b). The pattern of *CRG-L2* expression, high in tumors, but normally expressed in testis and placenta, resembles expression patterns of genes known as cancer-testis antigens (CT antigen). CT antigens are a group of genes classified by their exclusive expression in the testis and other reproductive tissues and diverse human cancers. The above findings suggest that *CRG-L2* is a potential CT antigen.

Discussion

With HCC on the rise worldwide and in the US, there is a clear need for more effective screening markers. Key characteristics of an effective HCC marker include a significant expression change in comparison with normal liver, deregulation in early stages of hepatocarcinogenesis, lack of response to proliferation caused by noncancerous conditions, limited expression in normal tissues, and the ability to be detected in body fluids. We have demonstrated that *CRG-L2* possesses many of these mentioned qualities of an effective and specific marker.

CRG-L2 *is upregulated early during tumor development but is not responsive to changes in proliferation*

By RT–PCR and *in situ* hybridization, we show increased expression of *CRG-L2* in murine liver tumors and in preneoplastic foci. Increased expression was also observed in a human hepatocellular adenoma and five HCC. *CRG-L2* mRNA was not increased in regenerating or newborn livers in the C3H/HeJ mice, indicating that *CRG-L2* is not responsive to changes in proliferation. Since cirrhosis and hepatitis are a result of noncancerous proliferation and inflammation within the liver, this may indicate that *CRG-L2* expression will be increased specifically in livers with HCC but not in livers with hepatitis or cirrhosis. To examine this hypothesis, several noncancerous livers with cirrhosis and hepatitis will need to be examined. *CRG-L2* was found to be increased in 55% of preneoplastic foci from 20-week-old DEN-treated C3H/HeJ mice. To our knowledge, this is the first robust marker for these early foci. CRG-L2 was upregulated in 69% of the 32-week tumors. This percentage of *CRG-L2* positive foci and tumors is high when compared to the current HCC marker. *AFP*, which was found in only 30% of preneoplastic foci from 20-week old DEN-treated C3H/HeJ mice. These findings suggest that *CRG-L2* may be useful in identifying early lesions, which is a weakness in all current screening techniques. We note that one of the human HCCs had extremely high levels of *CRG-L2* mRNA.

CRG-L2 *may be a CT antigen with a role in the extracellular matrix*

The examination of *CRG-L2* expression revealed that *CRG-L2* mRNA is expressed at very low levels in all normal tissues except in the mouse testis and human placenta. Therefore, *CRG-L2* may fall into a class of genes designated as CT antigens. The characteristics of CT antigens are a lack of expression in normal tissues, except reproductive tissues, and high levels of expression in a wide range of tumor types. Currently, there are more than 10 genes identified that are CT antigens, one of which, PAGE, also shows high expression levels in the placenta (Brinkman *et al*., 1998). Most CT antigens map to the X chromosome, but *SCP-1* (Türeci *et al*., 1998), *CT9* (Scanlan *et al*., 2000), and *OY-TES-1* (Ono *et al*., 2001) map to other chromosomes, as does *CRG-L2*. CT antigens are intriguing therapeutic targets for immunotherapy because of their limited expression in normal tissues and the fact that the testis and placenta are immune-privileged sites. However, the biological function and the relation to malignancy of most of these genes is unknown (Ono *et al*., 2001; Scanlan *et al*., 2002). Similarly, we do not yet know the biological function of CRG-L2. The predicted protein structure suggests that CRG-L2 belongs to a family of olfactomedin-related proteins, which includes olfactomedin, myocilin/TIGR, noelin-1, and hGC-1. Olfactomedin-related genes have characteristic tissue-restricted expression patterns suggesting a specialized function for each protein (Richards *et al*., 1998; Zhang *et al*., 2002). Based on tissue localization of several olfactomedin family members and the function of TIGR/myocilin, it is possible that olfactomedin-related proteins play an important role in protein–protein interactions within the extracellular matrix (Kulkarni *et al*., 2000). CRG-L2 also contains two collagen domains: proteins that contain collagen domains are also often involved in the structure of the extracellular matrix. Therefore, it can be speculated that CRG-L2 may play an important role in extracellular structure or intercellular signaling.

Our results clearly indicate that the expression of *CRG-L2* is increased in tumors. This increased expression in tumors and restricted pattern of expression in normal tissues suggest that CRG-L2 is a tumor-specific antigen. This alone would suggest that further studies of the usefulness of CRG-L2 as a clinical marker are warranted. However, the most useful clinical marker would be detectable without having to resort to tissue biopsies. If a patient displays an immunogenic response to a tumor-specific antigen, then the marker can be detected using blood samples, allowing more cost-effective screening of a larger number of high-risk patients. Not all tumor-related antigens are immunogenic or trigger an effective immune response in tumor patients (Rainov *et al*., 1995; Gilboa, 1999; Struss *et al*., 2001). Therefore, we are currently screening for the presence of antibodies to CRG-L2 in cancer patient sera.

In summary, the early deregulation of *CRG-L2* during liver tumorigenesis and its restricted expression in normal tissues suggest that CRG-L2 may be a promising new marker for hepatocellular carcinomas. Studies are under way to address the frequency at which CRG-L2 is deregulated in HCC and the mechanisms that lead to its upregulation.

Materials and methods

RACE

Rapid amplification of cDNA ends was performed in both directions using the SMART cDNA amplification kit (Clontech) from mouse liver tumor polyA RNA. 5′ and 3′ RACE were performed using the gene-specific primers, GSP-970 [5′-GCATGGCAAGAACAGACTGG-3′] and GSP-1241 [5′-GGATGAGAAGGGCATCTGGA-3′]. 5′ and 3′ RACE products that were identified with the corresponding GSP primer were gel extracted and cloned into TOPO-TA vector (Invitrogen). Cloned products were sequenced by Big Dye (ABI) in the McArdle Laboratory Sequencing Facility.

RNA Analysis

For the analysis of murine *CRG-L2* mRNA, total RNA was extracted from liver using guanidine thiocyanate/CsCl as described previously in Lukas *et al*. (1999). PolyA mRNA was isolated from 250 μg of total RNA using Oligotex mRNA Kit (Qiagen). RT–PCR was performed as described previously (Graveel *et al*., 2001) with primers RDA-3a [5′-CAACAACCTGGCTTAGAGC-3′] and RDA-3b [5′GCCATCTGATGCTCTATCC-3′].

For Northern blot hybridization, polyA RNA sample (2 μg) were prepared and electrophoresed as described previously (Lukas *et al*., 1999). Gel was soaked in five volumes of water for 5 min and then transferred overnight to a GeneScreen (NEN Life Science Products) membrane in 10 × SSC. Membrane was UV crosslinked twice (120 mJ) and baked in a vacuum for 2 h at 80°C. Membrane was prehybridized at 42°C overnight in a hybridization solution [50% formamide. 5 × Denhardt′s solution, 1% SDS, 10% dextran sulfate, 1 mg sonicated salmon sperm DNA (boiled). 5 × standard saline phosphate with EDTA (SSPE)]. Probes were labeled by nick translation (Rigby *et al*., 1977). The CRG-L2 fragment (188–1243) was released with *Eco*RI from the pCR-TOPO4 vector. ^{32}P-labeled probe was added to the hybridization buffer and hybridized overnight at 42°C. Blots were washed at room temperature in 2 × SSPE for 30 min and at 65°C for 45 min in 2 × SSPE, 2%SDS. Signals were visualized by autoradiography or phosphoimagery.

For the analysis of *CRG-L2* in human tissue, RT–PCR was performed for 25 cycles with primers hCRGL2a [5′-CATGGCAAGAACAGACTGGG-3′] and hCRGL2b [5′-GCCAGGAAACATCCCAAACTC-3′] and 10 μl of the reaction was electrophoresed on a 1% agarose/EtBr gel. The gels were soaked in 1 × TAE for 5 min, denatured for 30 min (1.5 M NaCl, 0.5 M NaOH), and neutralized for 30 min (1.5 M NaCl, 0.5 M Tris (pH 7.2), 1 mM EDTA (pH 8.0)). DNA was transferred to a Hybond N membrane (Amersham) with 20 × SSPE overnight. The membrane was baked for 30 min at 80°C in a vacuum oven and UV crosslinked twice (120 mJ). The membrane was prehybridized at 42°C for 3 h in a hybridization solution [50% formamide, 5% Denhardt's, 3.4 × SSPE, 10% dextran sulfate, 5% SDS, 1% sarkosyl, 100 mg sonicated salmon sperm DNA (SSS), 100 mg boiled SSS]. Probes were labeled by nick translation (CRG-L2 fragment, nucleotides 188–1243) and added to the hybridization solution. Membranes were hybridized overnight at 42°C and were washed for 20 min at room temperature in 2 × SSPE, 01% SDS, and for 2 h at 65°C in 0.5 × SSPE, 0.2% SDS. Signals were visualized by autoradiography and phosphoimagery. All primers used in this study were synthesized at the UW Biotechnology Center.

In situ hybridization

In situ hybridization was performed as described previously (Micales and Lyons, 2001) with the CRG-L2 plasmid 5-2 (containing nucleotides 82–1243) and AFP plasmid (containing nucleotides 726–1401) in the plasmid pCR4-TOPO (Invitrogen). Sense and antisense probes were synthesized using T7 or SP6 with a MAXIscript kit (Ambion) to generate ^{35}S uridine triphosphate (UTP)-labeled riboprobes. Hybridized sections were exposed to emulsion (NTB-2; Eastman Kodak) in the dark for 2 weeks before developing. After they were developed, the sections were counterstained with hematoxylin mounted, and viewed under both light-field and dark-field illumination.

Multiple tissue cDNA panel

The mouse and human tissue cDNA panels (Clontech) were screened following the manufacturer's instructions. After 28 cycles, 5 μl aliquots were removed at various time points. The mouse panel was screened with primers, GSP-970 and GSP-1241 (see RACE section for primer sequences), and the human panel was screened with primers hCRGL2-269 [5′-AGGGCCCACCAGGGCAGAAG-3′] and hCRGL2-479 [5′-ACATGCTTGGCTGCCGAGGG-3′].

Human tissue

Human tissue and serum was procured from the University of Wisconsin Surgical Pathology department, National Disease Research Interchange, and the NCI Cooperative Human Tissue Network. All samples analysed were primary tissues. As required by our IRB protocol, the identity of the patients was unknown. The excess tissue was frozen after surgery and stored at −70°C.

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