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Anterior Gradient-2 is Over Expressed by Fibrolamellar Carcinomas

Perumal Vivekanandan, Ph.D., Shien T.L. Micchelli, M.D., and Michael Torbenson, M.D.

Department of Pathology, Johns Hopkins University School of Medicine

Abstract

Anterior Gradient-2 expression is critical in normal embryonic development. Aberrant expression of Anterior Gradient-2 in adult tissues has been linked to breast, prostate, esophageal, and pancreatic carcinoma. To define the role of Anterior Gradient-2 in primary hepatocellular neoplasms, we used tissue microarrays and examined protein expression in typical hepatocellular carcinomas (N=44), fibrolamellar carcinomas (N=12), and hepatic adenomas (N=9). In non-neoplastic liver tissues, Anterior Gradient-2 was expressed in the septal-sized bile ducts and weakly in zone 3 hepatocytes in 11/61 (18%) of cases. In tumors, Anterior Gradient-2 was over expressed by only 1/44 (2%) of hepatocellular carcinomas. In contrast, 6/8 (75%) of fibrolamellar and 3/4 (75%) metastatic fibrolamellar carcinomas were positive. All 9 hepatic adenomas were negative. Further analysis of mRNA in fibrolamellar carcinomas identified 2 novel splice variants, but expression levels were very low. Sequencing of the *Anterior Gradient-2* gene in fibrolamellar carcinomas identified several polymorphisms (refSNP Ids: rs6842, rs8071, rs1051905) but no mutations. In conclusion, Anterior Gradient-2 is over expressed in the majority of fibrolamellar carcinomas but only rarely is over-expressed in hepatocellular carcinomas.

Introduction

Anterior Gradient-2 (*AGR2*) is located on Chr:7p21.3 [1] and encodes for the human homologue of a secreted protein first identified in *Xenopus laevis*. In *Xenopus*, the *AGR2* homologue is critical in forming anterior structures during embryonic ectoderm development [2]. The function of *AGR2* in human tissue is unknown, but *AGR2* mRNA remains expressed in the mature stomach, small intestine and colon of mice [3]. In the small intestine of mice, expression is found predominately in paneth, neuroendocrine, and goblet cells [4]. Mice with mutant *AGR2* have functional abnormalities of intestinal goblet cells and develop diarrhea [5].

Aberrant *AGR2* expression has been found in an esophageal carcinoma cell line[4], and in primary breast, lung, and prostate carcinomas [4,6–8]. *AGR2* can inhibit the function of P53 [9] and over-expression of *AGR2* in breast epithelial cell lines leads to metastases in an animal model [10]. However, the ways in which *AGR2* contributes to neoplasia is not restricted to over-expression, as loss of *AGR2* expression has also been associated with the dysplasia-to-carcinoma sequence in colonic polyps [11].

Please address correspondence to: Michael Torbenson, MD, Room B314, 1503 East Jefferson, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, Phone: 443-287-4730, Fax: 410-502-5158, Email: mtorben@jhmi.edu.

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The potential role for AGR2 in hepatocellular carcinoma has not been investigated. In this study, we sought to explore the normal expression pattern of AGR2 in the non-neoplastic liver and to characterize AGR2 expression in primary hepatocellular neoplasms including typical hepatocellular carcinomas, fibrolamellar carcinomas, and hepatic adenomas. AGR2 was found to be over-expressed in fibrolamellar carcinomas and to further study mechanisms that may contribute to over-expression, the coding region of the gene was sequenced and expression of splice variants was studied.

Materials & Methods

This study was performed with appropriate Institutional Review Board approval on de-identified tissues.

Protein expression and tissue microarrays

To investigate the expression of AGR2 protein in hepatic tissues, previously described tissue microarrays [12] were used. Following heat antigen retrieval, five micron sections were immunostained with an antibody to AGR2 (Abcam, Cambridge, MA, polyclonal IgG, 1:250 dilution). The Dako EnVision+ Peroxidase kit was used for immunostaining. Cases were scored as positive when at least 5% of hepatocytes were immunolabeled. Staining distributions for positive cases were scored on a scale of 0–3: 0 (from 0–4% of cells positive), 1 (from 5–25%), 2 (26–50%), 3 (51–100%). Intensity was graded on a scale of 0–3. Benign colon served as a positive control.

The tissue arrays contained eight primary fibrolamellar carcinomas from six women and two men with an average age at resection of 27 ± 13 years. In addition, 4 metastatic fibrolamellar carcinomas were studied from two men and two women. Two of the metastasis corresponded to primary tumors in the study while the other two did not have the primary tumor available. All individuals in which fibrolamellar carcinomas arose were Caucasian. The background livers showed no significant fibrosis or inflammation, as is typical of fibrolamellar carcinomas.

The tissue microarray slides contained hepatocellular carcinomas that arose in the setting of cirrhosis. However, because fibrolamellar carcinomas arise in the setting of non-cirrhotic livers [13], we also chose to use tissue microarray slides that were enriched for typical hepatocellular carcinomas that arose in non-cirrhotic livers. All together, the arrays for this study contained 44 primary hepatocellular carcinomas with paired non-neoplastic tissues. The tumors were from primary liver resections in 28 men and 16 women with an average age at resection of 57.9 ± 16.5 years. The underlying liver diseases were available in 35 individuals and included no known underlying liver disease with no significant fibrosis (N=16), chronic viral hepatitis C cirrhosis (N=10), chronic hepatitis B cirrhosis (N=2), cryptogenic cirrhosis (N=4), and alcohol related cirrhosis (N=3).

As a second control group, tissue microarrays of hepatic adenomas were also studied. These primary liver tumors also arise in non-cirrhotic livers with no evidence of background liver disease. This group also served as a control for any potential association with estrogen receptor expression and AGR2 positivity, an association that has been identified in breast carcinomas [7,10]. Hepatic adenomas typically arise in the setting of excess estrogen exposure and can express estrogen receptors [14]. Nine hepatic adenomas were studied and all arose in women with no background liver disease and had an average age of 34.2 ± 9.0 years at tumor resection.

Identification of CpG islands in AGR2 promoter

The promoter of *AGR2* [5] was analyzed for evidence of a CpG island using Methprimer (<http://www.urogene.org/methprimer/index1.html>), a publicly available web based program that can identify CpG islands.

Tissues for mRNA studies and for sequencing of the coding region of AGR2

For examination of mRNA, additional cases were studied based on available tissues. Fresh tissues were obtained at the time of surgery from primary liver neoplasms and adjacent non-neoplastic liver tissues. All tissues were obtained between 1999 through 2007 from routine surgical cases at the Johns Hopkins Hospital. Tissues were frozen in liquid nitrogen, and stored at -80°C prior to use. The pathological diagnoses were confirmed in all cases. The studied frozen tissues were from primary fibrolamellar carcinomas and metastases. Two cell lines were also examined, HepG2 and Hep3B. These cell lines were purchased (American Type Culture Collection, Manassas, VA) and grown in minimal essential medium under routine conditions. To normalize mRNA expression in the two cell lines, we used the average expression levels of five non-neoplastic, non-inflamed and non-cirrhotic livers.

RNA was extracted using TRIzol (Invitrogen life technologies, Carlsbad, CA, USA) followed by precipitation with isopropyl alcohol. cDNA was prepared using one μg of RNA with oligo dT primers and the Superscript First –Strand synthesis system for RT-PCR (Invitrogen life technologies, Carlsbad, CA, USA).

Primers were designed using the Genebank cDNA transcript NM_006408 to amplify exons 1–8 from cDNA. All primers are shown in Table 1.

Transcript expression by reverse transcriptase-PCR—cDNA was synthesized using 1 μg of RNA as input. One μl of cDNA was used then used as input for PCR. Primers were designed using the Genebank cDNA transcript NM_006408, with the forward primer in the exon 1 and the reverse primer in exon number 8 (Table 1). With this assay, an un-spliced transcript would produce a 653 bp product and any internal splice variation would be seen as smaller bands on agarose gels. An additional splice variant, termed the “long-form”, that uses alternative codons at the 5 primer end has been reported [5] and reverse transcriptase-PCR was also performed for this transcript (Table 1). PCR cycling conditions were 10 minutes at 95°C followed by 45 cycles of 95°C for 20 seconds, 55°C 25 seconds, and 72°C for 1 minute. The amplified products were analyzed by ethidium bromide in 1% agarose gels. Experiments without RT polymerase showed no amplicons. Selected bands were purified from the gel and directly sequenced.

AGR2 expression analysis by real time PCR—Two smaller transcripts were identified in this study by reverse transcriptase-PCR (termed AGR2 Δ 4-6 and AG Δ 6). The relative expression levels of these transcripts were further studied by real time PCR with assays that amplified only the smaller sized transcripts. To ensure specificity of the real time PCR assays for the smaller splice variants, the full length transcript was cloned and the real time PCR assays for the smaller sized transcript were negative using the full length transcript as template (data not shown).

Real time PCR was performed with the SmartCycler system (Cepheid) using the Fast Start SYBR green master mix (Roche) with 1 μg of cDNA and cycling conditions of 95°C for 10 minutes followed by 40 cycles of 95°C for 20 seconds, 55°C 30 seconds, and 72°C for 30 seconds. The specificity of PCR products were ascertained by melt curve analysis and agarose gel electrophoresis. Expression levels were normalized to beta-glucuronidase using previously described primers[15].

RESULTS

AGR2 protein expression in the non-neoplastic livers of the tissue microarrays

In the non-neoplastic livers, septal-sized bile ducts were typically positive (Figure 1A), while smaller ducts were negative. In addition, 11/61 (18%) of the benign livers showed weak immunostaining, generally limited to zone 3 hepatocytes (Figure 1B).

AGR2 protein expression in hepatocellular tumors

In tumors, AGR2 was positive in 6/8 (75%) of primary fibrolamellar carcinomas (Figure 1C) and 3/4 (75%) metastatic fibrolamellar carcinomas. The staining was cytoplasmic with no nuclear accumulation evident. The pale bodies present in fibrolamellar carcinomas were often strongly stained by AGR2 (Figure 1C). The median distribution of staining in fibrolamellar carcinomas was 2, and the median intensity of staining was 2. The staining patterns were as follows for the distribution (D) and intensity (I): D1I1 (N=1), D1I3 (N=1), D2I3 (N=2), D3I3 (N=2), D3I2 (N=1). For the two cases with paired primary and metastases, both the primary and metastases were AGR2 positive. In addition, five of the primary tumors had paired non-neoplastic tissues and in three of these cases the background livers showed weak hepatocellular staining as described above.

In one interesting case, a fibrolamellar carcinoma was present adjacent to a separate nodule of a more typical hepatocellular carcinoma in the same liver of a 26 year old woman with no background liver disease. AGR2 was positive only in the fibrolamellar tumor and negative in the separate tumor with typical hepatocellular carcinoma morphology.

In contrast to the fibrolamellar carcinomas, most typically hepatocellular carcinomas were negative for AGR2 (Figure 1D), while only 1/44 (2%) hepatocellular carcinoma was positive for AGR2 protein expression. The AGR2 positive hepatocellular carcinoma showed a typical compact growth pattern and arose in a 40 year old woman with no underlying liver disease. In one additional case, a hepatocellular carcinoma arose in the setting of cryptogenic cirrhosis and showed an unusual mixed hepatocellular-cholangiocellular morphology: the cholangiocellular component was positive for AGR2 while the hepatocellular component was negative. All of the nine hepatic adenomas were negative for AGR2 expression.

Statistical analysis (SYSTAT, V10) showed that AGR2 positivity was strongly associated with fibrolamellar carcinomas (Pearson Chi-square, $p < 0.0001$).

Promoter and mRNA analysis

In silico analysis of AGR2 promoter: Because AGR2 is normally expressed during embryogenesis, we investigated whether this gene was potentially inactivated by methylation in normal adult liver tissues. Many human genes are inactivated by methylation of CpG dinucleotides that cluster into CpG islands and can be located within gene promoters. The 2000 bases upstream of AGR2, which are reported to contain the promoter[5], showed no evidence for CpG islands.

mRNA analysis: As expected, AGR2 was over expressed in fibrolamellar carcinomas (Table 2). The AGR2 mRNA transcripts were next studied by reverse transcriptase PCR and two different splice variants were identified (Table 2). Sequencing of the variants showed one lacked exon 6 ($\Delta 6$ transcript) and one lacked exons 4–6 ($\Delta 4-6$ transcript) (Figure 2). The $\Delta 6$ transcript was present in the majority of the tumors and metastases while the $\Delta 4-6$ transcript was found in a single case (Table 2). Both used alternative splice sites. The 20 base pairs surrounding the $\Delta 4-6$ transcript spliced splice site are as follows: exon 4 5'-AGACATATGAAGAAGCTCTA/spliced/ATGTTTGTGACCCATCTCT-3' exon 7; while

the 20 base pairs surrounding the $\Delta 6$ transcript are as follows: exon 5 5'-TTGTCCTCCTCAATCTGGTT/spliced/ACCCATCTCTGACAGTTAG-3' exon 7. Quantitation by real time PCR showed expression levels of both transcripts were low, less than 1% of the total AGR2 expression levels (data not shown).

An additional splice variant, termed the “long form” has been previously reported [5]. PCR analysis for this form were negative in all tissues.

Sequencing of AGR2 exons—To further understand why AGR2 was over-expressed in fibrolamellar carcinomas, we next sequenced the exons 1–8 of AGR2. Five primary fibrolamellar carcinomas, 5 metastases, and the HepG2 and Hep3B cell lines were examined. Several polymorphisms were found (Table 3) but no mutations were identified.

DISCUSSION

In this study, AGR2 protein expression was strongly associated with fibrolamellar carcinomas but not typical hepatocellular carcinomas. This observation underscores the differences between fibrolamellar carcinomas and hepatocellular carcinomas and links fibrolamellar carcinomas to a unique biomarker that is not shared by most hepatocellular carcinomas.

To further understand why AGR2 was over-expressed in fibrolamellar carcinomas, mechanisms relevant to gene expression were studied. We investigated whether this gene had a CpG island within its promoter region, for which differential methylation could explain its aberrant expression. However, no CpG island was identified. Aberrant splicing of genes has been associated with hepatocellular carcinoma [15] and to investigate whether over-expression of AGR2 was related to changes in splicing, reverse transcriptase PCR was performed. Novel splice variants were found, but subsequent quantitative analysis showed expression of these splice variants was at very low levels, even though they were unique to tumors. Whether splice variants could also potentially be detectable in typical hepatocellular carcinomas was not further investigated because we found little evidence of protein expression by tissue microarrays. We also sequenced the coding regions of AGR2 and identified several polymorphisms but found no mutations. Together, these findings suggest AGR2 is most likely over-expressed because it is a target of a larger signaling/metabolic pathway. However, other possibilities such as translation dysregulation or abnormalities in protein degradation remain to be investigated.

The significance of the polymorphisms identified in the fibrolamellar carcinomas is uncertain. The polymorphism T498C (refSNP ID: rs:6842) was present in all but one of the fibrolamellar carcinomas and the majority of these cases were homozygous with a C/C genotype. This observation may be of interest, given that the frequency in fibrolamellar carcinomas appears higher than that in the NCBI database for the general Caucasian population, where 16.7% are reported to have a C/C genotype. However, the small numbers of cases in this study preclude drawing any conclusions on this point. Also, no change in amino acid sequence is predicted, making this SNP of uncertain relevance to AGR2 expression.

Within the hepatic tumors we studied, AGR2 expression was strongly associated with fibrolamellar carcinomas. Little is known about the signaling or metabolic pathways that are associated with AGR2 expression, but MAPK/ERK signaling can regulate AGR2 expression in cell lines in the context of physiological stress [16]. This observation may be relevant to fibrolamellar carcinomas in that we have previously shown that MAPK signaling is a feature of the fibrolamellar carcinoma transcriptome [17]. However, more definitive data on how AGR2 fits into the pathogenesis of fibrolamellar carcinomas are lacking at this time. In addition to data showing MAPK and PI3K signaling in fibrolamellar carcinomas [17], recent studies

have also shown that the mTOR pathway can be dysregulated in a subset of these tumors[12]. There is data from other cell types that these pathways can “cross-talk” [18], but the ways in which these pathways interact in fibrolamellar carcinomas are unclear. It may be of interest to note, however, that both the PI3K and MAPK pathways can converge on mTOR in the context of autophagy signaling.

The potential role for AGR2 role in maintaining neuroendocrine lineage in small intestinal cells [4] is particularly interesting given that fibrolamellar carcinomas can express chromogranin and other neuroendocrine markers [13]. A previously identified protein over-expressed in fibrolamellar carcinomas, neurotensin [19], has also been linked to neuroendocrine cells of the intestine [20].

In normal adult tissues, AGR2 is expressed in gastrointestinal organs in paneth cells, endocrine cells, and goblet cells. The role of AGR2 expression in these cell types is poorly understood, but in the small intestine a role for determining cell lineage fate has been suggested [4]. In this study, we extend the known expression pattern of AGR2 to include the septal sized bile ducts of the liver. We have not studied the largest of the intrahepatic bile ducts nor the extrahepatic biliary tree so the full expression pattern of AGR2 in the biliary system remains to be investigated. In addition, we found AGR2 expression in zone three hepatocytes in a subset of cases. One intriguing possibility would be that expression is related to maintaining the zonal gradients in the liver. However, another possibility would be that expression was stimulated due to microenvironmental stressors, as studies have shown that AGR2 can be up-regulated in response to stressors such as nutrient and oxygen deprivation[16].

In conclusion, AGR2 can be expressed in non-neoplastic liver tissues including septal-sized bile duct and zone 3 hepatocytes. The majority of fibrolamellar carcinomas (both primary and metastatic) over-express AGR2 protein, while hepatocellular carcinomas and hepatic adenomas are typically negative. Over expression of AGR2 protein does not appear to be associated with genetic or epigenetic changes within the AGR2 gene and is thus most likely over-expressed as part of a larger pathway.

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Figure 1.

Panel A, 100X original magnification. AGR2 showed weak expression in zone 3 hepatocytes in the background livers of 18% of cases. Panel B, 100X original magnification. Septal sized bile ducts were also positive for AGR2 in the non neoplastic liver tissues. Panel C, 40X original magnification. A fibrolamellar carcinoma shows strong cytoplasmic staining for AGR2. Immunostaining also highlights pale bodies, cytoplasmic inclusions that are often present in fibrolamellar carcinomas. Panel D, 40X original magnification. A hepatocellular carcinoma with typical morphology is negative for AGR2 expression.

Figure 2.

The full length reference cDNA and splice variants of AGR2 are shown. WT, full length reference transcript; LF, long form (see materials and methods); Delta 4–6, transcript missing exons 4 through 6; Delta 6, transcript missing exon 6.

Table 1

Primers used to study AGR2.

Assay	Primer name	Primer sequence, 5' to 3'	Amplicon size
Splice variant analysis	AGR2 1F	CGACTCACACAAGGCAGGT	653
	AGR2 1R	TCCACACTAGCCAGTCTTCTCA	
Long Form	AGR2 Longform 1F	GCCAACAGACAACCCAAAGT	241
	AGR2 Longform 1R	TCTGTTTGCAAGTGTTCCTCA	
Real time PCR, all variants	AGR2 Real Time 3F	TTTGTGACCCATCTCTGACA	173
	AGR2 Real Time 3R	GACAGACAGAAGGGCTTGGA	
Real time PCR, $\Delta 4-6$ transcript	AGR2 Real Time $\Delta 4-6$ F	TCCAGTGTGAGCATTCTTGC	190
	AGR2 Real Time $\Delta 4-6$ R	TGGGTCAACAAACATTAGAGCTT	
Real time PCR $\Delta 6$ transcript	AGR2 Real Time $\Delta 6$ F	AGACAAGCAACAAACCCTTGA	149
	AGR2 Real Time $\Delta 5$ R	CTGTCAGAGATGGGTAACCAGA	
Sequencing of exons	AGR2 1F	CGACTCACACAAGGCAGGT	653
	AGR2 1R	TCCACACTAGCCAGTCTTCTCA	
	AGR2 2F	TCTCCTGATGGCCAGTATGTC	582
	AGR2 2R	GAACCTGTGGTGTGGTGAGA	

Table 2
Clinicopathological information and slice variant analysis of fibrolamellar carcinomas and control cell lines

Case No.	Age/Race/Gender	AGR2 Δ6 transcript	AGR2 Δ4–6 transcript	Tumor AGR2 expression levels ¹
Primary tumors				
1N ² /1T ³	42/W/M	–/–	–/–	11.2
2N/2T	26/W/F	–/–	–/–	9.3
3N/3T	17/W/F	–/+	–/–	9.4
4N/4T	28/W/F	–/+	–/+	4.5
5T ⁵	12/W/F	NA ⁴ /–	NA/–	6.9
Metastatic Tumors⁵				
1	14/W/F	+	–	11.2
2	20/W/F	+	–	11.1
3	33/W/F	+	–	13.2
4	28/W/F	+	–	13.6
5	27W/F	–	–	8.3
Cell lines⁵				
HepG2		+	–	2.7
Hep3B		+	–	0.7

¹ Log 2 fold change of tumor vs normal for total AGR2 transcripts

² N, non-neoplastic liver tissues

³ T, Tumor

⁴ NA, Tissue not available

⁵ Non-neoplastic paired tissues were available for the first four primary tumors. For tumors without paired normal tissues (case 5T and the metastases), expression levels were normalized to the average expression from the four non-neoplastic livers from cases 1–4. For cell lines, expression was normalized to a pool of 5 non-neoplastic, non-cirrhotic livers.

Table 3

Base pair changes found in Exons 1 through 8 of AGR2 in fibrolamellar carcinomas and cell lines. rs6842 refSNP ID: rs8071 refSNP ID: rs1051905

Case No.	T498C refSNP ID: rs6842	A735T refSNP ID: rs8071	C809T refSNP ID: rs1051905
Primary tissues			
1N [*] /1T ^{**}	heterozygous	wild type	wild type
2N/2T	wild type	wild type	wild type
3N/3T	homozygous	homozygous	wild type
4N/4T	homozygous	wild type	wild type
5N/5T	homozygous	wild type	wild type
Metastatic Tumors			
1	homozygous	wild type	wild type
2	homozygous	wild type	wild type
3	heterozygous	wild type	wild type
4	heterozygous	heterozygous	wild type
5	homozygous	wild type	wild type
Cell lines			
HepG2	wild type	wild type	heterozygous
Hep3B	wild type	wild type	heterozygous

* N, non-neoplastic liver tissues

** T, Tumor