

RAPID COMMUNICATION

Mannose 6-Phosphate/Insulin-Like Growth Factor 2 Receptor (M6P/IGF2R) Variants in American and Japanese Populations

J. Keith Killian,^{1,2} Yoshihiko Oka,^{1,3} Hong-Seok Jang,^{1,4} Xialong Fu,^{1,5} Robert A. Waterland,¹ Tetsuro Sohda,⁶ Seigo Sakaguchi,³ and Randy L. Jirtle^{1,2*}

¹Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina

²Department of Pathology, Duke University Medical Center, Durham, North Carolina

³Department of Gastroenterology, Fukuoka University Chikushi Hospital, Fukuoka, Japan

⁴Department of Radiation Oncology, Uijongbo St. Mary's Hospital, Uijongbo, Korea

⁵Cancer Hospital/Cancer Institute, Shanghai Medical University, Shanghai, China

⁶Third Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka, Japan

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M6P/IGF2R encodes a multifunctional protein involved in lysosomal enzyme trafficking, fetal organogenesis, tumor suppression, and cytotoxic T cell-induced apoptosis. M6P/IGF2R is imprinted and expressed only from the maternally inherited allele in marsupials and rodents. In contrast, humans were initially reported to differ from the imprinted mammalian orders by not having an imprinted M6P/IGF2R; however, some studies now suggest M6P/IGF2R imprinting may be a human polymorphic trait. Mutational and functional evidence are consistent with M6P/IGF2R also being a tumor suppressor in human colon, liver, lung, breast, and ovarian cancers. M6P/IGF2R expression is also pathologically downregulated following mammalian *in vitro* embryo culture, resulting in fetal overgrowth and “large offspring syndrome.” Therefore, the M6P/IGF2R imprint status in humans is an unresolved question that critically impacts upon biological issues ranging from human cancer predisposition to evolution. Attempts to further characterize the imprint status of human M6P/IGF2R and loss of heterozygosity at this locus in cancer have been hindered by a lack of readily usable polymorphisms. To facilitate these genetic analyses, we have screened American and Japanese populations for M6P/IGF2R single nucleotide polymorphisms (SNPs). We have identified nine novel SNPs intragenic to human M6P/IGF2R, and have described experimental conditions for their optimal use. Three identified amino-acid variants in the M6P/IGF2R ligand-binding domains may be under selection in humans. *Hum Mutat* 18:25–31, 2001. © 2001 Wiley-Liss, Inc.

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DATABASES:

IGF2R – OMIM: 147280; GDB: 120083; GenBank: Y00285 (cds), AF348209 (genomic);

<http://www.GeneImprint.com> (The Genomic Imprinting Website)

INTRODUCTION

The human M6P/IGF2R gene (IGF2R; MIM# 147280), located at 6q26-27 [Laureys et al., 1988; Rao et al., 1994], encodes for a multifunctional receptor that possesses distinct binding sites for several classes of molecules, including phosphomannosyl glycoproteins and insulin-like growth factor II (IGF2) [Jirtle,

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*Correspondence to: Randy L. Jirtle, Departments of Radiation Oncology and Pathology, Duke University Medical Center, Durham, NC 27710. E-mail: jirtle@radonc.duke.edu

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1999a]. The M6P binding site of this receptor mediates lysosomal enzyme trafficking, latent TGF-beta activation, and cell-mediated cytotoxic cell death [Jirtle, 1999b; Motyka et al., 2000]. M6P binding is the most ancient function attributed to this receptor, and it is present in invertebrates [Lakshmi et al., 1999], fish [Nadimpalli et al., 1999], amphibians and birds [Clairmont and Czech, 1989], and mammals [Killian et al., 2000]. The viviparous mammalian M6P/IGF2R also contains an independent binding site for IGF2; however, the marsupial homologue binds IGF2 with a 70-fold lower affinity than that of eutherian mammals. This difference in binding efficiency has been traced to repeat domain 11 of the receptor [Jirtle, 1999b; Killian et al., 2000]. Rather than provide an intracellular signal promoting cell growth, M6P/IGF2R internalizes and delivers bound IGF2 to the lysosomal compartment for degradation. Receptor acquisition of an IGF2 binding site occurred following the divergence of therian mammals (i.e. marsupials and true placental mammals) from the egg-laying monotreme mammals and non-mammalian vertebrates [Killian et al., 2000]. Thus, the history of M6P/IGF2R evolution reveals that its M6P binding function predates that for IGF2 by at least 300 million years.

M6P/IGF2R plays a critical role in embryonic development, immunity, and tumor suppression. M6P/IGF2R deficiency during murine development is associated with cardiac abnormalities, cleft palate, fetal overgrowth, and perinatal lethality [Lau et al., 1994; Ludwig et al., 1996; Wang et al., 1997; Melnick et al., 1998]. Recently, in an experimental model of "large offspring syndrome," Young et al. [2001] identified epigenetic changes intragenic to M6P/IGF2R that correlate with decreased gene expression and fetal overgrowth in livestock progeny which had been cultured in vitro during pre-implantation development. Consistent with the receptor's role in regulating cell growth and tissue proliferation, genetic analyses reveal frequent M6P/IGF2R loss of heterozygosity in liver, lung, breast, and ovarian cancer [De Souza et al., 1995; Hankins et al., 1996; Kong et al., 2000; Rey et al., 2000], and M6P/IGF2R is a common target of microsatellite instability in gastrointestinal tumors [Souza et al., 1996; Ouyang et al., 1997].

Interestingly, the M6P/IGF2R has been identified as a novel target of autoantibodies in pa-

tients with autoimmune diseases [Tarrago et al., 1999]. Furthermore, the M6P/IGF2R facilitates T cell activation by internalizing CD26/DPPIV (dipeptidyl peptidase IV), a cell surface T cell activation antigen [Ikushima et al., 2000]. Other recent findings indicate that Granzyme B internalization by M6P/IGF2R is required for cytotoxic T cells to induce apoptosis [Motyka et al., 2000]. The characterization of M6P/IGF2R allelic variants, including those that are non-synonymous and alter the protein product, will facilitate analysis of genetic linkage between immunopathologies and M6P/IGF2R.

M6P/IGF2R is expressed only from the maternally inherited allele in members of the marsupial [Killian et al., 2000] and rodent lineages [Barlow et al., 1991; Hu et al., 1998; Mills et al., 1998] while egg-laying animals are not imprinted at this locus [Killian et al., 2000; Nolan et al., 2001]. These observations are consistent with an ancestral mammalian origin of M6P/IGF2R imprinting roughly 150 million years ago; alternatively, M6P/IGF2R imprinting may have evolved convergently. Intriguingly, the human M6P/IGF2R is reported to have diverged from the imprinted mammalian orders such that individuals inherit two functional alleles [Kalscheuer et al., 1993; Ogawa et al., 1993; Smrzka et al., 1995; Riesewijk et al., 1996]; however, other reports suggest that imprinting at this locus is a polymorphic trait in humans with a minority population expressing only one copy of this gene [Xu et al., 1993; Xu et al., 1997; Riesewijk et al., 1998]. Polymorphic imprinting of M6P/IGF2R is predicted to predispose people to cancer because of the inheritance of a haploinsufficient tumor suppressor gene [Xu et al., 1997]. Recessive mutations and/or iatrogenic epimutations affecting embryonic and post-natal development would also show increased penetrance in imprinted individuals who have no recourse to a functional wild type allele.

The essential question of whether some humans inherit an imprinted M6P/IGF2R is still unresolved because allelic expression analysis is hindered by a dearth of characterized polymorphisms within the coding sequence. We have herein identified nine novel human M6P/IGF2R polymorphisms to further facilitate such studies, and have compiled them into a table with those previously characterized. We also have described experimental conditions for their op-

timal use, and have discussed those polymorphic variants prone to artifactual results.

MATERIALS AND METHODS

DNA Samples

Blood samples for DNA genotyping were obtained from healthy adults at Duke University Medical Center in the United States and Fukuoka University Hospital in Japan. Use of these samples was approved by the Duke University Medical Center Institutional Review Board. Genomic DNA was extracted from blood according to the manufacturer’s protocol (QIAamp DNA Blood Kit, QIAGEN Inc., Valencia, CA).

Human M6P/IGF2R SNP Identification

The 48 individual *M6P/IGF2R* exons with approximately 100 bp flanking intronic sequence were PCR-amplified from 12 individuals using oligonucleotides previously described [Killian and Jirtle, 1999]. PCRs were performed with 50–100 ng template DNA, 1.5 U Platinum Taq DNA polymerase (Life Technologies, Baltimore, MD), 15 pmol primers, 1.5 mM MgCl₂ and 100 μM dNTPs in a 30 μL PCR reaction volume (94°C × 15 sec, 55°C × 5 sec, and 72°C × 45 sec for 30–35 cycles). Amplimers were analyzed and extracted from 2% agarose gel (GenElute, Sigma, St. Louis, MO), and sequenced either manually (Thermo Sequinase, USB, Cleveland, OH) or on the ABI Prism 377 using BigDye terminators (PE Biosystems, Foster City, CA). SNPs were identified as sequence dimorphisms in the sequenced DNA or di-allelic homozygous alternates.

Human M6P/IGF2R SNP Analysis

PCR primers for amplification of the 10 *M6P/IGF2R* SNPs are given in Table 1. The allele frequencies of these 10 polymorphisms were determined in a total of 93 individuals, including 43 American (25 white American, 16 black Ameri-

can, and two Asian American) and 50 Japanese individuals. PCRs were performed with 50–100 ng template DNA, 1.5 U Platinum Taq DNA polymerase, 15 pmol primers, 1.5 mM MgCl₂ and 100 μM dNTPs in a 30 μL PCR reaction volume (94°C × 15 sec, 55°C × 5 sec, and 72°C × 45 sec for 30–35 cycles). Genotypes were confirmed by DNA sequencing. The distribution of allelic variants among American and Japanese populations was analyzed by chi-square tests. To decrease the likelihood of alpha-error associated with multiple testing, only differences with *P* < 0.01 were considered statistically significant.

RESULTS

The population-specific frequency of one previously identified [Zhong et al., 1999], and nine novel *M6P/IGF2R* single nucleotide polymorphisms (SNPs) are provided in Table 2. Allele frequencies were derived from analysis of 50 Japanese and 43 Americans (16 black, 25 white, two Asian). No statistically significant differences in allelic distribution were found between black and white Americans, so these populations were pooled in subsequent analyses. Comparing the American and Japanese samples, highly significant differences in allelic distribution were found for six of the 10 SNPs. The polymorphisms c.1197A > G, c.1737G > A, and c.2286G > A, have been described as silent mutations in ovarian cancer [Rey et al., 2000]. Our analysis shows that these variants represent common polymorphisms in the human population, and suggests no particular relationship to ovarian cancer. Two of the 10 polymorphisms are transversions (c.901C > G and IVSx47–5A > T), while the remainder are transitions.

DISCUSSION

We have identified novel allelic variants of the human *M6P/IGF2R*, and have shown that

TABLE 1. PCR Primers for Analysis of Human *M6P/IGF2R* SNPs

SNP	Location	F primer	R primer
c. 901C>G	Exon 6	CTAAGGGTACTGTGATTATCACTC	GAAAGTCAGGTCCTTGCTGGAG
IVS8-22G>T	Intron 7	GTGGAAAATCTGCATTAAGCTGCATG	CCTTCTCCTAAGCAGCGCC
c. 1197A>G	Exon 9	GACTAAGTAAGACTGTAATCTTCTAATACC	CGCACAGAGGTTGTTGACGTAC
c. 1737A>G	Exon 12	GTGACTCAGAGAAATGAGCATTGC	CTAACTCATTCCAAACTGGATGCC
c. 2286A>G	Exon 16	GTGACTCCTCACGTCGCTCACG	CACAGGCATGAGTATCCTCAGG
IVS23-42C>T	Intron 22	CTGCACTGTGCTTGTGGGCTGC	GACTCTTGACCGGCCTCTCAGTTC
c. 5002A>G	Exon 34	GAAATTGATGGTCTGACTTGCG	GCACTGGAGATGCACTTCTCC
c. 6206A>G	Exon 40	GCATAGACACAGTGACAGTCTGATC	GCAGTCTGAAGTTCACATGC
IVS47-107A>G	Intron 46	CCATGCCCTCTCTACACTGGAG	CCTGATGAGAACGACATGGACAGC
IVS47-5A>T	Intron 46	CCATGCCCTCTCTACACTGGAG	CCTGATGAGAACGACATGGACAGC

TABLE 2. Allelic Variants of Human M6P/IGF2R

Variant	Amino acid change	Allele	American			Japanese			P value
			n	(%)	Frequency	n	(%)	Frequency	
c. 901C>G (Exon 6)	<u>CTG>GTG</u> Leu>Val	CC	32	(74.4)	C=0.8721	28	(56.0)	C=0.7500	0.086
		CG	11	(25.6)	G=0.1279	19	(38.0)	C=0.2500	
		GG	0	(0)		3	(6.0)		
IVS8-22G>T (Intron 7)		GG	1	(2.3)	G=0.1977	12	(24.0)	G=0.5100	7 × 10 ⁻⁵
		GT	15	(34.9)	T=0.8023	27	(54.0)	T=0.4900	
		TT	27	(62.8)		11	(22.0)		
c. 1197A>G (Exon 9)	<u>TCA>TCG</u> Ser	AA	24	(55.8)	A=0.6744	2	(4.0)	A=0.2400	2 × 10 ⁻⁷
		AG	10	(23.3)	G=0.3256	20	(40.0)	G=0.7600	
		GG	9	(20.9)		28	(56.0)		
c. 1737A>G (Exon 12)	<u>GGA>GGG</u> Gly	AA	7	(16.3)	A=0.3023	30	(60.0)	A=0.7800	5 × 10 ⁻⁸
		AG	12	(27.9)	G=0.6977	18	(36.0)	G=0.2200	
		GG	24	(55.8)		2	(4.0)		
c. 2286A>G (Exon 16)	<u>ACA>ACG</u> Thr	AA	12	(27.9)	A=0.5116	3	(6.0)	A=0.2500	0.002
		AG	20	(46.5)	G=0.4884	19	(38.0)	G=0.7500	
		GG	11	(25.6)		28	(56.0)		
IVS23-42C>T (Intron 22)		CC	2	(5.0)	C=0.2250	7	(14.0)	C=0.3500	0.203
		CT	14	(35.0)	T=0.7750	21	(42.0)	T=0.6500	
		TT	24	(60.0)		22	(44.0)		
c. 5002A>G (Exon 34)	<u>AGG>GGG</u> Arg>Gly	AA	0	(0)	A=0.1512	1	(2.0)	A=0.1700	0.647
		AG	13	(30.2)	G=0.8488	15	(30.0)	G=0.8300	
		GG	30	(69.8)		34	(68.0)		
c. 6206A>G (Exon 40)	<u>AAC>AGC</u> Asn>Ser	AA	38	(90.5)	A=0.9524	22	(44.0)	A=0.6500	1 × 10 ⁻⁵
		AG	4	(9.5)	G=0.0476	21	(42.0)	G=0.3500	
		GG	0	(0)		7	(14.0)		
IVS47-107A>G (Intron 46)		AA	20	(47.6)	A=0.6429	6	(12.0)	A=0.4000	8 × 10 ⁻⁴
		AG	14	(33.3)	G=0.3571	28	(56.0)	G=0.6000	
		GG	8	(19.1)		16	(32.0)		
IVS47-5A>T (Intron 46)		AA	5	(11.6)	A=0.2558	8	(16.0)	A=0.4000	0.059
		AT	12	(27.9)	T=0.7442	24	(48.0)	T=0.6000	
		TT	26	(60.5)		18	(36.0)		

Polymorphisms in the coding region and introns are numbered according to Morgan et al. [1987], with nomenclature based on Antonarakis [1998]. Intronic polymorphisms are amplified with the published primers for the nearest exon [Killian and Jirtle, 1999]. Note the presence of non-synonymous polymorphisms in exon 6 (c. 901C>G, Leu252Val); exon 34 (c. 5002G>A, Gly1619Arg); and exon 40 (c. 6206A>G, Asn2020Ser). P value refers to the significance of statistical comparison between American and Japanese allele frequencies at each SNP locus.

they are present in both the American (black and white) and Japanese populations, although at different frequencies (Table 2). Because these polymorphisms are shared by these populations, they were possibly present prior to the geographic separation of ancestors common to blacks, whites, and Japanese. Alternatively, the variants could have originated after the divergence of these populations with subsequent introgression. Because of the abundance of SNPs with population-specific frequencies, the *M6P/IGF2R* may serve as a model gene for future analyses of human relatedness and diasporas. Comparison of the combined American versus Japanese populations reveals that six out of 10 SNPs have significantly different allele frequencies ($P < 0.01$). These SNPs include IVS8-22G>T, c.1197A>G, c.1737A>G, c.2286A>G, c.6206A>G, and IVS47-107A>G.

Interestingly, the *M6P/IGF2R* variant manifesting the least difference between Americans and

Japanese is the non-synonymous c.5002A>G. The American and Japanese populations analyzed in this study both contained 30% heterozygosity at this locus. However, when we split the American population by race, we noted a discrepancy in c.5002A>G heterozygote frequency, with 12.5% and 36% heterozygosity in black and white, respectively ($P = 0.098$). Analysis of native African and European populations would help elucidate whether c.5002A>G is ancestral to humans, or whether it has arisen convergently in one or more populations, with subsequent introgression into the black American gene pool.

It is striking that three out of six of the polymorphisms we identified within the human *M6P/IGF2R* coding region are non-synonymous. While c.901C>G, Leu252Val is a relatively conservative amino acid change, c.5002G>A, Gly1619Arg, and c.6206A>G, Asn2020Ser variants differ substantially. The c.901C>G, Leu252Val resides in repeat domain 2 of the

M6P/IGF2R protein, a domain reported to be involved in M6P binding [Jirtle, 1999b]. The c.5002G>A, Gly1619Arg falls within repeat domain 11 of the protein, which contains the IGF2 binding site [Jirtle, 1999b]. Finally, c.6206A>G, Asn2020Ser is within repeat domain 13, which reportedly is an IGF2 affinity-enhancing domain [Jirtle, 1999b].

Although c.901C>G, Leu252Val (exon 9) is not a radical alteration, there is strict conservation of leucine at this position in the alignment of platypus, opossum, mouse, rat, and cow M6P/IGF2R [Killian et al., 2000]. Because this amino acid variation occurs in the M6P binding region, it may affect functions including lysosomal enzyme trafficking, TGF- β activation, and cytotoxic T-cell mediated tumor cell apoptosis.

Concerning c.5002G>A, Gly1619Arg, we also note strict conservation of glycine at this position in platypus, opossum, mouse, rat, and cow [Killian et al., 2000], suggesting that Arg1619 may indeed have a functional consequence in humans. Because of its positioning in the IGF2-binding repeat domain 11, the functional consequence of inheriting a heterozygous Gly1619Arg M6P/IGF2R complement may affect IGF2 binding. Because fetal growth is largely controlled by M6P/IGF2R-mediated IGF2 degradation, the IGF2-binding domain variants of human M6P/IGF2R are potentially involved in offspring size in humans. The possible involvement of c.5002G>A in human pathology requires further linkage and functional studies. Interestingly, Glycine 1619 is conserved in platypus, an animal whose M6P/IGF2R reportedly does not bind IGF2 [Killian et al., 2000].

The third non-synonymous polymorphism in the M6P/IGF2R coding sequence, c.6206A>G, Asn2020Ser (exon 40), occurs at a relatively variable position in the mammalian comparison [Killian et al., 2000]: platypus and opossum have an aspartic acid, mouse and rat a glutamic acid, and cow an asparagine. Elucidating the roles of these protein-altering M6P/IGF2R variants in human traits requires biochemical investigation.

Experimentally, while most M6P/IGF2R polymorphisms clearly distinguished the parental alleles, there are some precautions. The c.2286A>G and c.5002G>A polymorphic sites in exons 16 and 34, respectively, show band compressions when sequenced manually with radio-labeled terminators, i.e., Thermo Sequenase. This requires the

use of "GC-rich" sequencing protocols, including substituting deoxyinosine for deoxyguanosine when DNA sequencing. These compression artifacts are not observed using the ABI automated sequencer with BigDye terminators.

There are two previously described polymorphisms in the distal end of the 3' UTR that are particularly problematic for PCR analysis: a GT dinucleotide repeat polymorphism [Goto et al., 1992] and a tetranucleotide insertion/deletion [Hol et al., 1992]. The GT repeat is prone to the usual Taq DNA polymerase slippage artifacts during PCR, and the results can be further obscured by the presence or absence of the tetranucleotide insertion/deletion that is only 27 bp away. The PCR primers initially reported by Goto et al. [1992], and utilized in some studies of human M6P/IGF2R imprinting, in fact hybridize directly to the tetranucleotide insertion/deletion, such that primer binding is dependent upon the status of the ACAA insertion/deletion polymorphism. Results are therefore often difficult to interpret when the 3' UTR GT-repeat polymorphism is used for parental allelic analysis. In contrast, we invariably obtain unambiguous and reproducible results when samples are characterized using the SNPs reported herein.

Regardless of which polymorphism is employed, we have observed frequent stochastic amplification of one variant over another whenever more than one round of PCR and/or greater than 35 cycles in a single round of PCR are used. If insufficient starting template is available to yield a robust amplicon in a single round of PCR, results must be confirmed by several repeat analyses, and preferably with more than one polymorphism.

To summarize our experience with M6P/IGF2R allelic analysis, we recommend using SNPs rather than the GT-repeat polymorphism for M6P/IGF2R allelic expression and LOH analyses. Starting template of sufficient quality and concentration should be used to ensure robust amplification in less than 35 total PCR cycles. In the case of small quantities of tissue or paraffin-embedded tissues in which this is not always possible, repeat analyses must be performed to rule out stochastic amplification. It is important to note that of the published papers addressing the issue of M6P/IGF2R imprinting in humans, deviation from biallelic expression has only been reported when using the ACAA

insertion/deletion and GT-repeat polymorphisms [Kalscheuer et al., 1993; Ogawa et al., 1993; Riesewijk et al., 1996; Smrzka et al., 1995]. Whether these reports will be supported by expression studies using these novel SNPs remains to be determined.

In conclusion, our identification and characterization of nine novel *M6P/IGF2R* SNPs is essential for researchers to address several questions pertaining to human development, cancer, and imprinting. These include: 1) determining whether some humans inherit a cancer-predisposing, imprinted *M6P/IGF2R*; 2) evaluating the role of *M6P/IGF2R* allelic loss in cancer patient prognosis and response to therapy; 3) determining the *M6P/IGF2R* mutation status in cancers from different ethnic populations; and 4) assessing the potential influence of human embryo culture procedures such as IVF on *M6P/IGF2R* expression. The availability of abundant, high-quality *M6P/IGF2R* polymorphisms will enable these important questions to be rapidly and conclusively resolved. Finally, just as *M6P/IGF2R* is a model gene for mammalian phylogenetic inference [Killian et al., 2001], we expect population analyses of *M6P/IGF2R* variants to be highly informative for studies of human evolution, relatedness, and diasporas.

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