The role of mannose-6-phosphate/insulin-like growth factor 2 (M6P/IGF2R) receptor in carcinogenesis: a review

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Abstract

The cation independent mannose 6-phosphate/insulin-like growth factor 2 (M6P/IGF2R) is a multifunctional receptor. It is involved in a variety of cellular processes which become disregulated in cancer. Its tumor suppressor role was recognized a long time ago. However, due to its multifunctionality, it is not easy to understand the extent of its relevance to normal cellular physiology. Accordingly, it is even more difficult understanding its role in carcinogenesis. This review presents critical and focused highlights of data relating to M6P/IGF2R, obtained during more than 25 years of cancer research.

1. Introduction

The cation independent mannose-6-phosphate/insulin-like growth factor 2 (M6P/IGF2R) is a multiple ligand - binding cell surface receptor, ubiquitously expressed in human tissues. Its truncated soluble form is present in the circulatory system (~0.7μg/mL) [1]. The human gene for M6P/IGF2R, considered to be a tumor suppressor, is located at 6q26, spread over approximately 137 kb of genomic DNA. It consists of 48 exons which make an open reading frame encoding a protein of 2491 amino acids (AA) [2]. The first 40 AAs represent a cleavable residue segment [3]. For this reason, some discrepancies relating to numbering the amino acids arose in the literature. For most researchers, methionine is the first AA that needs to be counted (M1). Other researchers count glutamine (Q41) as the first amino acid of the IGF2R protein sequence [3, 4]. This creates a certain amount of confusion, especially when it comes to the topographic localization of point mutations. For that reason, we have based this review on the comprehensive genomic and mRNA sequence of *M6P/IGF2R*, which are available in the GeneBank under accession numbers NG_011785 and NM_000876, respectively. In this review, we consider methionine, coded by nucleotides ATG and positioned at cDNA 149-151, the first amino acid in the M6P/IGF2R protein structure and we count the rest of amino acids accordingly. The structure of the protein, related to the *M6P/IGF2R* coding region, is shown in Figure 1. The protein repeats are shown to be consistent with the updated data on IGF2R structure, UniProtKB/Swiss-Prot, Accesion P11717. This structure differs slightly from the data, first published in1998, on IGF2R repeats borders, starting with repeat 13 [3].

2. M6P/IGF2R – Multifunctional receptor

The major part of the protein consists of a large extracellular domain (2264 AAs), a very short transmebrane domain (23AAs, 2305-2327), and a cytoplasmic domain that is 164 amino acids long (AAs 2328-2491), constituting the C-terminus [4]. The spacial organization of the extracellular domain (AAs 41-2304) creates 15 homologous extracellular repeats (134-167 AAs long) which represent 15 homologous structural units [3]. They display significant similarity in amino acid sequence and disulphide distribution (16-38%). This kind of structure already indicates multifunctional binding properties and, consequentially, multiple functioning. This includes the physiological processes of lysosomal enzyme trafficking, endocytosis and lysosomal degradation of extracellular ligands and regulation of apoptotic and mitogenic effects [5]. The significance of M6P/IGF2R can be best understood through the structure and roles of its ligands. Hence, it is not surprising that a majority of the research relating to M6P/IGF2R focuses on this part of its biological significance.

The M6P/IGF2R ligands' list is quite long and is divided into two general classes: those containing mannose-6-phosphate (M-6-P) and those that are mannose-6-phosphate free.

The targeting of newly synthesized hydrolases to the lysosome has been a well-recognized function of M6P/IGF2R. This action is possible because it „recognizes" the M-6-P residue on N-linked oligosaccharides which allows the process of hydrolase sorting. The receptor initially binds hydrolase in the Golgi. The release of hydrolases

from the receptor happens when acidity increases. This event takes place in the pre-lysosomal compartment. The next step is recycling of the receptor into the Golgi apparatus to repeat the process, or moving the receptor to the plasma membrane [6].

Some M-6-P bearing ligands, with well-known relevance to cancer, are leukemia inhibitory factor, proliferin and cathepsin D - a strong promoter of cancer cell proliferation and invasion which is included in lysosome biogenesis [7-9]. There are also some multi-protein complexes containing the latent TGF-β which binds to the membrane M6P/IGF2R, to be activated extracellulary [10].

Among M6P/IGF2R ligands without M-6-P, the most relevant to cancer may be insulin-like growth factor 2 [11], urokinase-type plasminogen activator receptor (uPAR) [12], retinoic acid [13], serglycin [14] and heparanase [15]. Recently, CREG (cellular repressor of E1A-stimulated genes 1) was also shown to be a M6P/IGF2R ligand. The consequence of their binding is cell growth suppression, probably through M6P/IGF2R-mediated CREG subcellular distribution [16].

Retinoic acid (RA) (with a still unknown binding site) and non-glycosylated peptide growth factor IGF2, may be the most-studied M-6-P free ligands, having major importance in both embryogenesis and carcinogenesis. There is a clear functional link between these two ligands: while IGF2 does not inhibit RA binding to M6P/IGF2R, the RA has been shown to stimulate IGF2 internalization [13].

Due to the fact that IGF2 binds to IGF1 and insulin receptor, in addition to M6P/IGF2R, its final influence on cell functioning clearly depends on the targeted receptor. Its growth-promoting effects are mediated by its binding to IGF1 and/or insulin receptors. On the other hand, IGF2 recognition and internalization through the

M6P/IGF2R has proved to be a general mechanism for modulating the IGF2 circulating levels through lysosomal degradation. The best evidence for this mechanism of action comes from gene targeting studies showing a fetal overgrowth, elevated levels of circulating IGF2, and perinatal lethality due to major cardiac abnormalities in M6p/Igf2r deficient mice [17,18]. Knocking out either M6p/Igf2 or Igf1r completely rescued this phenotype [19].

In vivo, even a modest increase of M6p/Igf2r, as recently shown in M6p/Igf2r transgenic mice expressing high levels of Igf2, causes a significant delay in the occurrence of breast tumor. At the same time, it decreases their multiplicity [20]. However, the first evidence of M6P/IGF2R's suppression function came far earlier, from *in vitro* studies.

3. Recognizing *M6P/IGF2R* as a tumor suppressor gene

The tumor suppressor function of M6P/IGF2R was initially proved in 1999. Transfection of a JEG-3 choriocarcinoma cell line with human *M6P/IGF2R* sense and antisense RNAs (nt 1-718) caused reduced *M6P/IGF2R* expression and a significant increase in cell growth rate. Both types of transfectants were injected into nude mice. As expected, the antisense transfectants induced significantly larger tumors in a higher proportion of animals as compared to sense transfectants [21]. The same type of experiment was performed in the opposite direction. The expression of exogenous wild-type *M6P/IGF2R* in SW48 colon cancer cells, originally containing mutated *M6P/IGF2R*

in poly$(G)_8$ region, induced a significant decrease in growth rate and an increased rate of apoptosis [22]. Several lines of evidence further supported these findings.

Transfection of MCF-7 cells with a ribozyme cleaving more than 40% of endogene M6P/IGF2R target, reduced both β-glucuronidase and the IGF2 internalization ~40%, induced the MCF-7 cells growth and decreased the apoptotic rate upon TNF treatment [23]. On the other hand, transfection of full length cDNA in *M6P/IGF2R* non-expressing mouse breast cancer cells 66cl4 did not decrease, surprisingly, their *in vitro* growth capacity and invasiveness. However, the ability of these cells to form tumors *in vivo* was seriously diminished as a result of their decreased growth rate [24].

Another experimental model, based on the transfection of full-length *M6P/IGF2R* cDNA into *M6P/IGF2R*-deficient lung cell carcinoma cell line SCC-VII, showed similar results. *M6P/IGF2R* expression established the restoration of intracellular retention and the processing of lysosomal cathepsins B-, D- and L (as contrasted with their pericellular accumulation in non-transfectants) which negatively impacted an anchorage-independent proliferation and invasiveness *in vitro* and tumor growth *in vivo* [25]. All these valuable studies have dealt with relatively narrow aspects of *M6P/IGF2R* functioning. The obtained results strongly support *M6P/IGF2R* as a suppressor gene. However, there is a very interesting study which shows that the M6P/IGF2R functioning should be considered at multiple levels. In a high percentage of LNCaP and PC-3 prostate cancer, its forced expression resulted in an increased cell number, probably due to an increased proliferation rate, although one could not exclude the possibility of decreased cell survival. The authors suggested that the IGF2 and M-6-P binding functions have opposite effects in the growth of these cells. As a cumulative result of specific binding,

proliferative or inhibitory signaling occurs [26]. This very intriguing, logical hypothesis still needs to be proved in other experimental models.

4. Mutations in cancer

Before 1995, when the first mutations of *M6P/IGF2R* were published in two different papers, it was well known that different malignant tumors (breast, ovary, melanoma, lymphoma, renal carcinoma) frequently display loss of heterozygosity (LOH) at 6q26-27, where *M6P/IGF2R* resides [27-32]. In 1995, as one might have expected based on previous findings, the first analyses focusing on LOH and mutation analyses in this particular gene were published.

4.1. Breast cancer

In the first study, based on 40 breast cancer samples (21 invasive carcinomas, 19 ductal carcinomas *in situ* (*DCIS*)), LOH was found in 12 samples, among them five carcinomas *in situ*. The *DCIS* LOH samples were further sequenced and two mutations were found, one in exon 31 (Gln1445His) and one in exon 40 (Thr2379Pro) [33]. In 1997, this finding was confirmed: LOH was detected in 4 out of 18 *DCIS* [34]. However, the suggestion that LOH of the *M6P/IGF2R* represents an early event in breast carcinogenesis was not supported by recent results obtained in a large study based on immunohistochemistry.

An overall and significant overexpression was shown in the high-grade *DCIS* (N=61) as compared to normal adjacent tissues [35]. On the other hand, a significant decrease of *M6P/IGF2R* in 24% of analyzed invasive breast carcinomas (N=133) supported the loss of its suppressing function in advanced, invasive breast tumors [35]. Based on these three reports, it is clearly not easy to determine the role of M6P/IGF2R receptor in the early steps of carcinogenesis in breast tissue.

Radiation alters the level of its mRNA, as shown in MCF-7, T47D (both ER-positive) and MDA-MB-231 (ER-negative) breast cancer cell lines, after being irradiated with 2 Gy. The mechanism for this phenomenon seems to be stabilization of the *M6P/IGF2R* transcript. The potential therapeutic clinical implications of this observation need to be explored [36].

4.2. Hepatocellular cancer (HCC)

In the second early study, LOH was shown in 11 of 16 informative HCC samples. Additional LOH was detected in one of three analyzed fibrolamellar tumors and two of [37]. While this last result strongly indicated that *M6P/IGF2R* LOH was an early event in liver carcinogenesis, the results of this study and several other studies also pointed out M6P/IGF2R dysfunction as a relatively common event in these types of tumors. This was not confirmed in only one study [38]. Although the authors [38] hypothesized that the *M6P/IGF2R* LOH may not be common in Japanese HCC patients, some other results obtained on a Japanese population do not support this hypothesis [39]. However, the research based on *M6P/IGF2R* in these specific tumors was, in fact, the extensive study

of *M6P/IGF2R* mRNA quantification in HCC. This study showed a 58% decrease of *M6P/IGF2R* mRNA and 27% of protein in 7 of 11 carcinomas, as compared with normal liver tissue. Hence, the mutations discovery was the result of a logical step forward [40]. Already in 1996, the tumors analyzed [37] were sequenced and mutations were detected in four tumor samples: three with proven LOH (Gly1449Val, Gly1449Glu and one sample with splice mutation) and two samples with retained wild-type allele (Gly1464Glu and one splice mutation) [41].

Loss of heterozygosity analysis was performed in five more studies, All together, these studies included 146 informative patients [37-39, 42-44]. In some cases, two or more separate analyses were performed on DNA extracted from different topological tumor sites and/or regenerative and dysplastic nodules (DS). For this reason, the number of heterozygous samples analyzed in these studies is bigger than the number of patients: 168 (145 HCC). The total number of LOH positive HCC samples was 79 (79/145, 54.5%). LOH was also present in a smaller proportion of dysplastic nodules (Table 1). This is a very interesting finding because dysplastic liver lesions are considered to be the HCC precursor.

The samples with LOH were further analyzed for the presence of mutations in three of these studies [38, 39, 43]. In Yamada's study, the amplified exons 27-28 (AAs 1224-1339) and 31 (AAs 1418-1481) were directly sequenced [43]. The targeting was well designed to explore the possibility of existence of M6P/IGF2R hot spots existence, because mutations in amino acids 1445, 1464 and 1449 were already known [33,41]. Mutations were present in six of 11 LOH positive tumors. These included the already known Gly1449Val in two samples, a new mutation Cys1262Ser in another sample and

frameshift mutations induced by „G“ deletion in poly$(G)_8$ repeat present in exon 28 (NM_000876: nucleotides 4089-4096) in three additional samples. This specific mutation will be discussed later.

In the second paper, mutations were present in 9 of 43 HCC LOH -ositive tumors, but not in the LOH-positive (3/4) DS [39]. In addition, dissected, phenotypically unchanged liver tissue adjacent to HCC showed LOH in two out of five samples. The analyzed region was far broader than in previous research as it included sequencing of exons 8-10 (AAs 295-438), 27-35 (AAs 1224-1722) and 37-40 (AAs 1773-2022). Hence, it was not surprising that the spectrum of discovered mutations broadened: Asp1268Asn, Met1625Leu, Pro1444Ser, Ser1628Phe, Gly1315Gln, His1878Gln, Thr1650Ile and an already known mutation consisting of „G“ deletion in the poly$(G)_8$ repeat stretch of exon 28. In order to understand the meaning of these mutations, one should be familiar with the specific functions of the receptor, relating to its „molecular anatomy“. The amino acids 1445, 1449 and 1464 belong to repeat 10 (AAs 1395-1532) (Figure 1). Contrary to expectations, this repeat is not the binding repeat domain for IGF2, nor it is considered the key repeat for M-6-P binding (Figure 1).

4.3. Other types of cancer

Prostate cancer loss of *M6P/IGF2R* mimics the HCC: among 36 informative patients, LOH was present only in malignant tumor tissues of 15 patients. It was also detected in high-grade PIN (prostatic intraepithelial neoplasia) which was present in 7 of

11 of these patients. This may indicate that LOH of *M6P/IGF2R* represents an early genetic event in prostate cancer [45].

It is surprising that there is only one mutational research study on *M6P/IGF2R* in lung cancer performed on paraffin-embedded sections [46]. It included 22 patients, all were smokers. Both LOH and mutational analyses were done through DNA sequencing. Among 19 heterozygous persons, LOH was present in 11 (11/19). On further sequencing (exons 8-11, 27-29, 31, 33-34, 37-39) of these 11 samples, six mutations were discovered, as shown in Table 1. Based on this study, it was not possible to estimate the importance of M6P/IGF2R in early lung carcinogenesis. However, we have noticed that two mutations considered to be substitution point mutations resulting in Gly1296Arg and Gly1564Arg substitutions, may, in fact, be splice-site mutations. In the first case, the coding triplet of nucleotides for glycine, GGT, changes into CGT. In the second case the glycine coding triplet GGG changes into CGG. In both cases, the codin triplets originate from two different exons. In both cases, the first coding nucleotide originates from the 5' (upstream) exon (27 and 33, respectively), while the remaining two nucleotides originate from the 3' (downstream) exons (28 and 34, respectively). We have analyzed these two mutations through the Human Splicing Finder and the program calculated the variation to be >10% [47]. Based on these results, we conclude, with confidence, that these two mutations affect the donor splice side. As a consequence, one would expect exon-skipping with unstable RNA/protein or the activation of a cryptic splice site leading to premature termination codon and non-sense-mediated mRNA decay. If this really happened, then it may be an additional explanation for the lack of M6P/IGF2R protein in the samples with these two mutations.

In vitro analysis performed on 22 different cancer cell lines that were resistant to growth inhibition by TGF-β, showed an aberrantly migrating band obtained by amplification of exon 40, originating from DNA extracted from lung adenocarcinoma cell line NMS-Lu3. It was further sequenced. Point mutation resulting in asparagine to serine substitution was detected at codon 2020 (Figure 1) [48].

In a subset of 25 laryngeal squamous cell carcinomas (all patients were smokers), LOH was found in only three samples. All of them were in an advanced stage and of high grade, strongly indicating LOH of *M6P/IGF2R* as a late event in laryngeal carcinogenesis [49]. This is in accord with findings obtained on adrenocortical tumors, where LOH was detected in 15 tumors, among them 11 were malignant. It did not, however, correlate with tumor expansion or metastatic potential [50]. The results of LOH analyses from several other studies are shown in Table 1 [51-55]. We have tried to collect and present all relevant data, but there is a possibility that we may have missed some.

4.4. Microsatellite instability and M6P/IGF2R mutations, the importance of specific polymorphisms

Genetic mutational studies had profiled a repetitive stretch of 8 "G" (poly$(G)_8$) nucleotides in coding region of *M6P/IGF2R* (exon 28) as a *locus minoris resistentiae*. These were always joined with tumors which demonstrate microsatellite instability (MI). The consequence of one or two base pairs insertion/deletion is the creation of premature stop codon. Although not considered as a Real Common Target for mutation occurrence in three different cancer types (colon, endometrium stomach), based on statistical meta-

analysis proposed by Woerner et al. (which was criticized later), the reported rate of mutation in this specific area is quite high, especially in colorectal tumors (10-28%), as shown in Table 2 [56, 57]. The importance of these specific mutations in this region has been well recognized and some recent studies focus on this region.

Unfortunately, due to the lack of stringent and well-described criteria that would define the MI status of the 148 sporadic breast cancer tumors in which mutation in this region was not found (based on results obtained in other tumor types one would expect that all, or most of these tumors were MI negative), it is not possible to include this study in the statistics of specific mutations for the purpose of this review [58]. However, the same study showed one "G" deletion in breast cancer cell lines CAL51 and MT-31 [58]. Based on available data, it seems that some 15% of MI positive tumors have mutation in poly$(G)_8$, which is quite high (Table 2) [59-64].

In addition, there is a stretch of variable number of „GT" repeats at the 3' prime of the gene (NG_011785, nt:141809-141840). According to two reports, this variability may relate to the increased susceptibility to both lung cancer and oral squamous cell carcinoma [65, 66]. It would not be surprising if a different number of „GT" repeats in 3'UTR changed the list of mi-RNA binding candidates, making them potential regulators of M6P/IGF2R activity. However, this hypothesis needs to be confirmed through strong experimental data. Our preliminary search of this region through several micro-RNA databases did not show any mi-RNA that would target the *M6P/IGF2R* mRNA in the area of these specific repeats [67].

Finally, some recent data indicates the importance of certain polymorphisms in *M6P/IGF2R* (rs998075, rs998074) as possible risk factors for osteosarcoma. It was

proposed that aberration of CpG methylation due to the presence of polymorphism induces changes in gene expression. However, this was not supported with functional data obtained at the bench [68]. On the other hand, through a comprehensive analysis (site-directed mutagenesis, qRT-PCR, cell cycle analysis, NMR spectroscopy) that also included bioinformatics, it was shown that the M6P/IGF2R polymorphism Gly1619Arg in repeating domain 11 has no effect on its structure, real-time ligand binding kinetics, protein half-life and its cellular distribution [69]. More multilevel research will be needed in order to understand the functional consequences of the *M6P/IGF2R* polymorphisms discovered so far. The same applies to mechanisms which regulate its activity.

5. Epigenetic regulation of *M6P/IGF2R* transcription

Some recent data points out very interesting epigenetic mechanisms that influence the *M6P/IGF2R* transcription. In mice, the gene is crucial for survival. Knock-out Igf2r mice were dying *in utero* around the time of birth [17]. Contrary to primates, including humans, where *M6P/IGF2R* expression follows biallelic mode of expression, the gene is maternally expressed (paternally imprinted) in rodents, marsupials and artiodactyls [70]. However, in a subset of individuals, human *M6P/IGF2R* expression can be predominantly or exclusively transcribed from the maternal allele [71].

In mice, the Igf2r gene codes two reciprocally - imprinted transcripts, due to two differentially-methylated, cytosine rich, regions (DMRs). While the first one (DMR1), includes promoter for maternally expressed sense Igf2r transcript, the second one (DMR2), positioned in the second intron of the Igf2r, includes promoter for paternally

expressed antisense transcript, Air [72]. Contrary to mice, human *M6P/IGF2R* contains only one DMR positioned in intron 2, and there are no antisense transcripts [73]. In addition to this CpG island, there are two more islands in humans: one located in its promoter region (usually unmethylated) and the other located in intron 44.

The mechanism of biallelic expression of *M6P/IGF2R* in a majority of humans was a subject of controversy in light of a well-known fact on the broad deviation of DMR methylation status [74]. It was estimated (based on a study that included 680 individuals), that 11% and 3% of people have an abnormally low or high level of DMR methylation on the paternal allele, respectively [75]. On the other hand, the data obtained from histone modifications analyses was consistent, as there is an equal enrichment of active histone modifications (acetylation of H3 and H4 and di- and tri-methylation of H3-Lys4 and H3-Lys9) in the promoter region on both parental alleles. In mice, on the other hand, there is a clear difference in histone modifications relating to a specific allele. Activating modifications were found only at the M6p/Igf2r promoter on the maternal allele, while the same enrichment on the paternal allele was localized only in the Air promoter region [76].

6. Mutation versus protein function

Intermolecular cross-linking of two M6P/IGF2R was originally shown on the binding of multivalent ligand, β-glucuronidase [77]. The mechanism of this interaction came from two more studies: the first one showed that dimerization occurs independently of ligand binding, while the results of the second study clearly showed that there is no

specific dimerization domain. Hence, oligomerization seems to be a result of multiple interactions that exist along the ectodomain of the receptor [78, 79]. The last data show that IGF2 binds independently to repeating domain 11 on each receptor monomer. On the other hand, M-6-P bearing ligand binding is bivalent, and this kind of binding requires cooperative interaction of cognate sites on both monomers of the dimeric receptor.

In humans, two IGF2 hydrophobic binding sites are located in repeating domain 11 (AAs 1533-1666) contributed by the residues in repeating domain 13 (AAs 1821 – 2008) and necessary for high affinity IGF2 binding (10^{-10} mol/L) [80, 81]. Site-directed studies have revealed IGF2 amino acids F48, R49, S50, A54 and L55 as critical for IGF2 binding to M6P/IGF2R. It was also shown that surface-exposed residues, not directly involved in binding itself, strongly influence the binding affinity [82]. In M6P/IGF2R, the residues Y1542, S1543, G1546, F1567, G1568, T1570, I1572, S1596, P1597 and P1599 have been considered as "the candidates" involved in this interaction [83]. Another study confirmed the crucial binding role for I1572 (profiled as crucial for this interaction already in 1999), and for F1567. These two amino acids seem to be strong candidates for anchoring interactions with F48 and L55 [84, 85]. Among *in vivo* occurring mutations that were studied in these models, three mutations were discovered in lung carcinoma (Gly1564Arg – discussed previously regarding the possibility of misinterpretation: point mutation vs. splice mutation, Ala1618Thr, Gly1619Arg) and they directly affect repeating domain 11. According to Brown, Gly1564Arg probably disrupts the secondary structure of the protein, while the effects of the other two mutations seem to be small [83].

In the model proposed in 2002, even-numbered domains face one direction, while the odd-numbered domains face the opposite direction. According to this model, the putative IGF2 repeating domain 11 is adjacent to repeating domain 13 which contains fibronectin type II-like insert [83].

On the other hand, there are three binding sites for M-6-P containing ligands. Two high affinity binding sites are localized in domains 1-3 (crystallography reveals that repeat 3 sits on the top of repeats 1 and 2), and 7-9. One low affinity site maps to domain 5 [84, 85]. The difference in binding capacity is possibly due to the absence of two cysteine residues which form a stabilizing disulfide bond in repeats 3 and 9 [86]. Site-directed mutagenesis studies have identified the core binding site residues in repeating domains 3 and 9 to be made of a glutamine, a glutamate, a tyrosine and an arginine residues [87, 88]. Finally, Olson's crystallographic study revealed that the receptor forms five three-repeat units with binding sites for IGF2 and M-6-P stand on the opposite face of the receptor [89].

Because most of mutations occur in the extracytoplasmic domain, it was very important to understand how these mutations alter the function of the protein.

6.1. Significance of specific mutations

The first functional analyses of the mutant *M6P/IGF2R*, published in 1999, were based on a mini receptor mutation construct. Mutant full-length *M6P/IGF2R* cDNAs were transfected into 293T cells and expressed as soluble receptor constructs [90, 91]. The specific five mutations tested in this system were: Gln1445His, Gly1449Val,

Gly1464Glu, Ile1572Thr, Cys1262Ser. Among all these amino acids, only the glycine at 1464. is not conserved in humans, rats, bovines and mice, while all other amino acids are. That fact itself indicates their importance in the M6P/IGF2R functioning.

These experiments revealed the importance of 1572. isoleucine. Its substitution with threonine completely abolishes the IGF2 binding. The mutants Cys1262Ser and Gly1449Val demonstrated a measurable reduction in ligand-receptor association (60% decrease). Glu1445His and non-conserved Gly1464Glu bound it with almost wild-type affinity. Regarding the underlying mechanism for reduced binding of both IGF2 and M-6-P by mutants Cys1262Ser and Gly1449Val, it was a surprising discovery that these two mutations decrease the number of binding sites, although both mutants are eventually capable of interacting with ligands. Several explanations were offered and all of them are based on a change in M6P/IGF2R conformation as a consequence of mutation.

7. Directly involved in cell signaling?

Finally, although its cytoplasmatic tail lacks a kinase domain, there are a number of older studies providing evidence for a signaling role as well as for interaction with heteromeric G-proteins [92-94]. It still needs to be discovered how the physiological concentrations of soluble M6P/IGF2R inhibits DNA synthesis in both hepatoma cells (BRL cells) and mice fibroblasts (3T3 cells). The same paper showed that the inhibitory effect on DNA synthesis can be reduced by the presence of IGF2 [95]. A similar effect was obtained with MDA-MB-231 cells stably transfected with full length *M6P/IGF2R* cDNA. In addition to decreased DNA synthesis, the tumor-forming capacity of these cells

was significantly diminished, as shown *in vivo* on athymic *nu/nu* mice. Finally, there was a 50% decreased level of IGF1-R phosphorylation, accompanied by reduced phosphorylation of ERK1/ERK2 in these cells [96]. Due to the fact that an increase in M6P/IGF2R decreases the bioavailability of IGF2, the silencing of IGF1R signaling pathway can be considered a logical consequence.

However, the latest published study relating to the possible signaling function of M6P/IGF2R, gave somewhat opposite results: *in vitro* siRNA down-regulation of *M6P/IGF2R* leads to a significant reduction in IGF2 (and not IGF1)-stimulated, heterotrimeric G-protein dependent, ERK1/ERK2 activation. This is in accord with the hypothesis that direct signaling through M6P/IGF2R receptor may exist [97]. The meaning of these opposite recent discoveries needs to be explored further. There may be a possibility that the difference obtained in these experiments reflects the genetic background of the cell models taken for these experiments (MDA-MB-231- invasive breast cancer cell lines vs. HEK293 - human embryonic kidney cell line). Also, the different methods that were used in these studies, cannot be entirely excluded as the reason for the obtained differences.

8. Conclusion

More than 10 years ago, it was proposed that there was a multifunctional involvement of M6P/IGF2R in cancer, starting with its role as a mediator of protease activity, its importance for TGF-β activation and an increase in IGF2 degradation [98]. Today, keeping in mind the importance of its extensive arrays of ligands, it is hard to

imagine the process related to carcinogenesis not including its role. Logically, and in the detail discussed in a recent review, there are many potential therapeutic applications relating to M6P/IGF2R [99]. So far, the most promising results are obtained with synthesized M6P analogues which may change the binding affinity of M6P/IGF2R. Hopefully, the development of potential drugs which will influence M6P/IGF2R activity and/or function in cancer, will become a reality. This will not be an easy task, primarily due to the selectivity needed. To succeed in targeting the IGF2 binding site without affecting the rest of receptor's activities would be an important and encouraging step forward.

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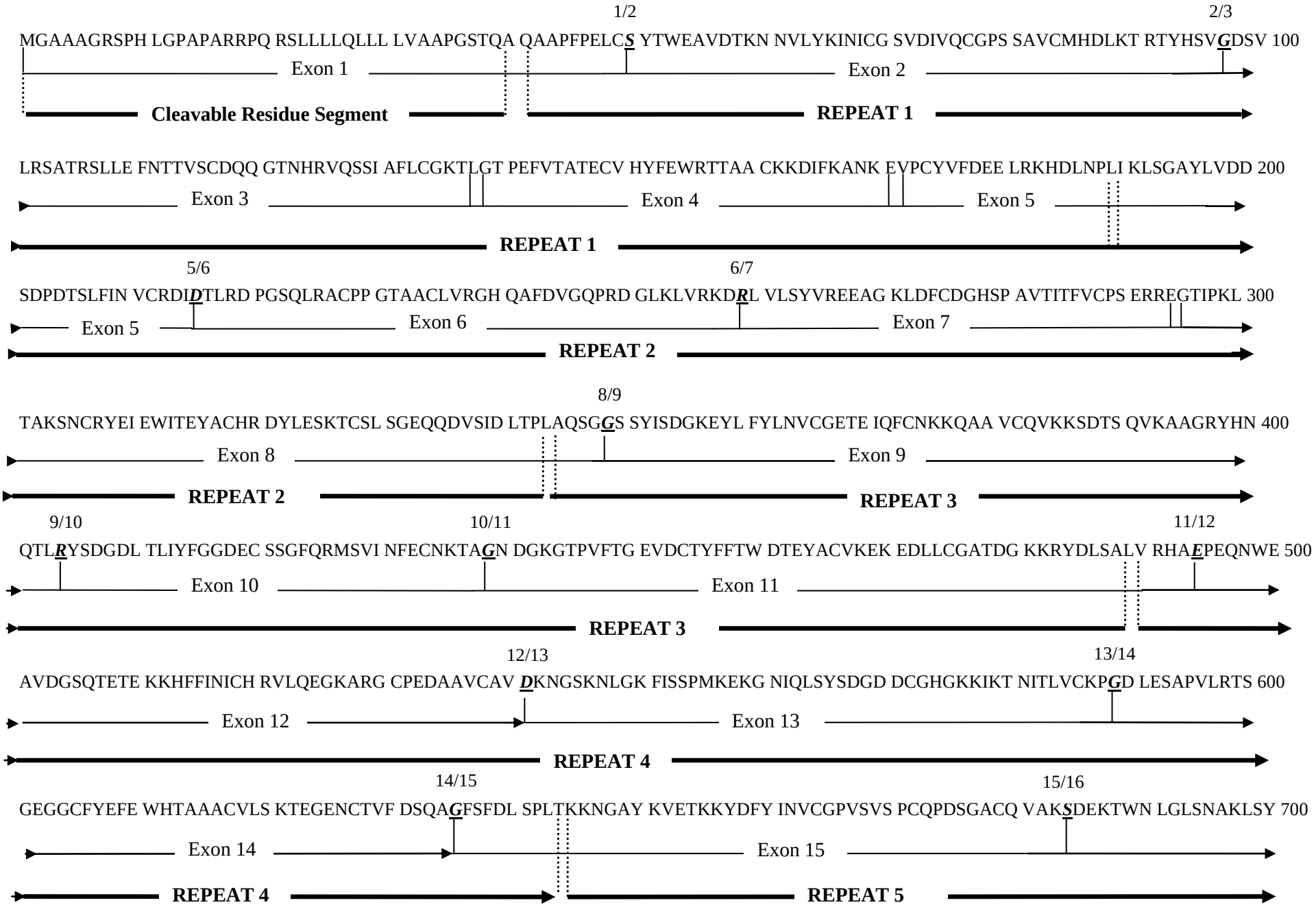
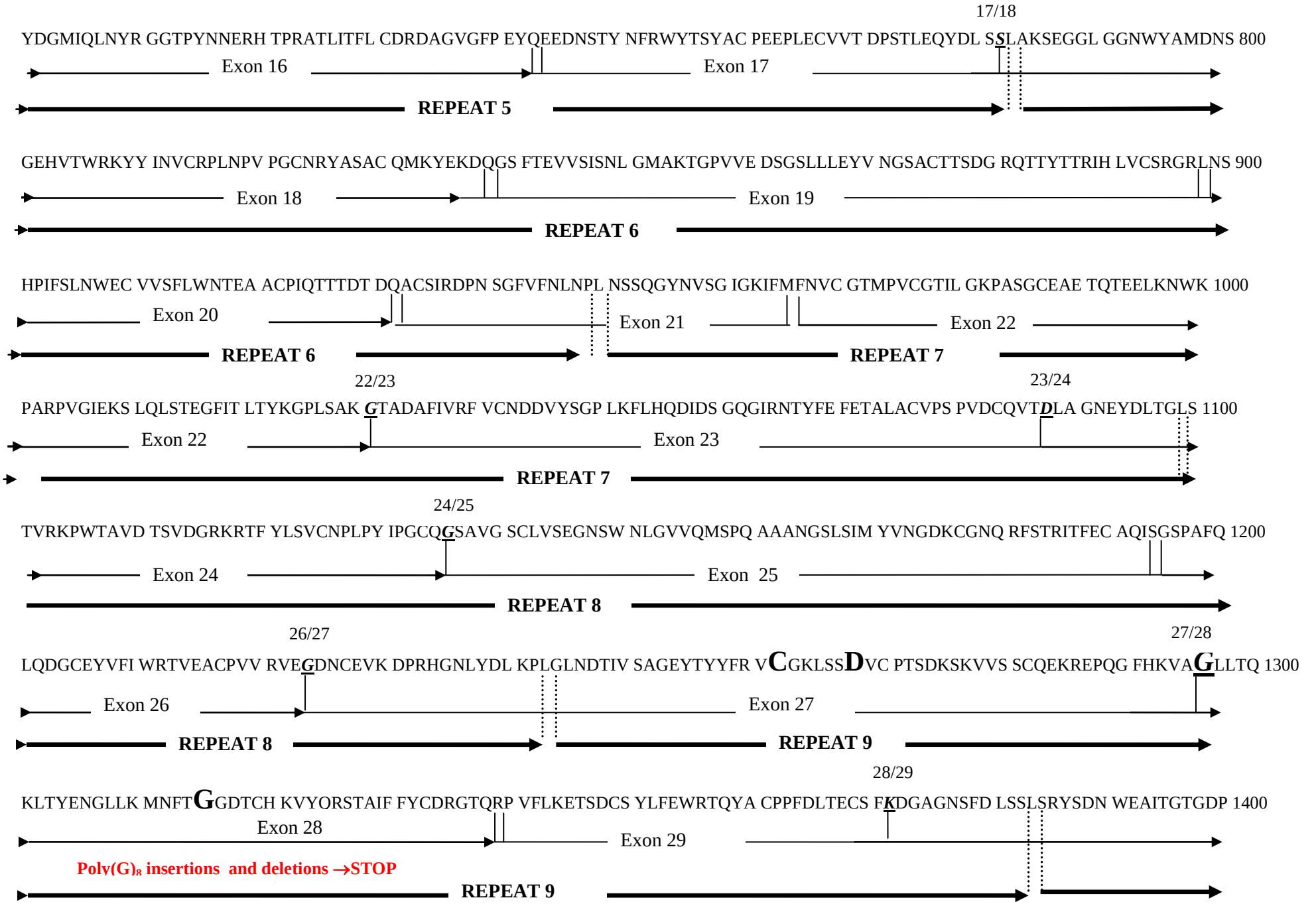
Figure 1. M6P/IGF2R: coding region and corresponding protein sequence




17/18
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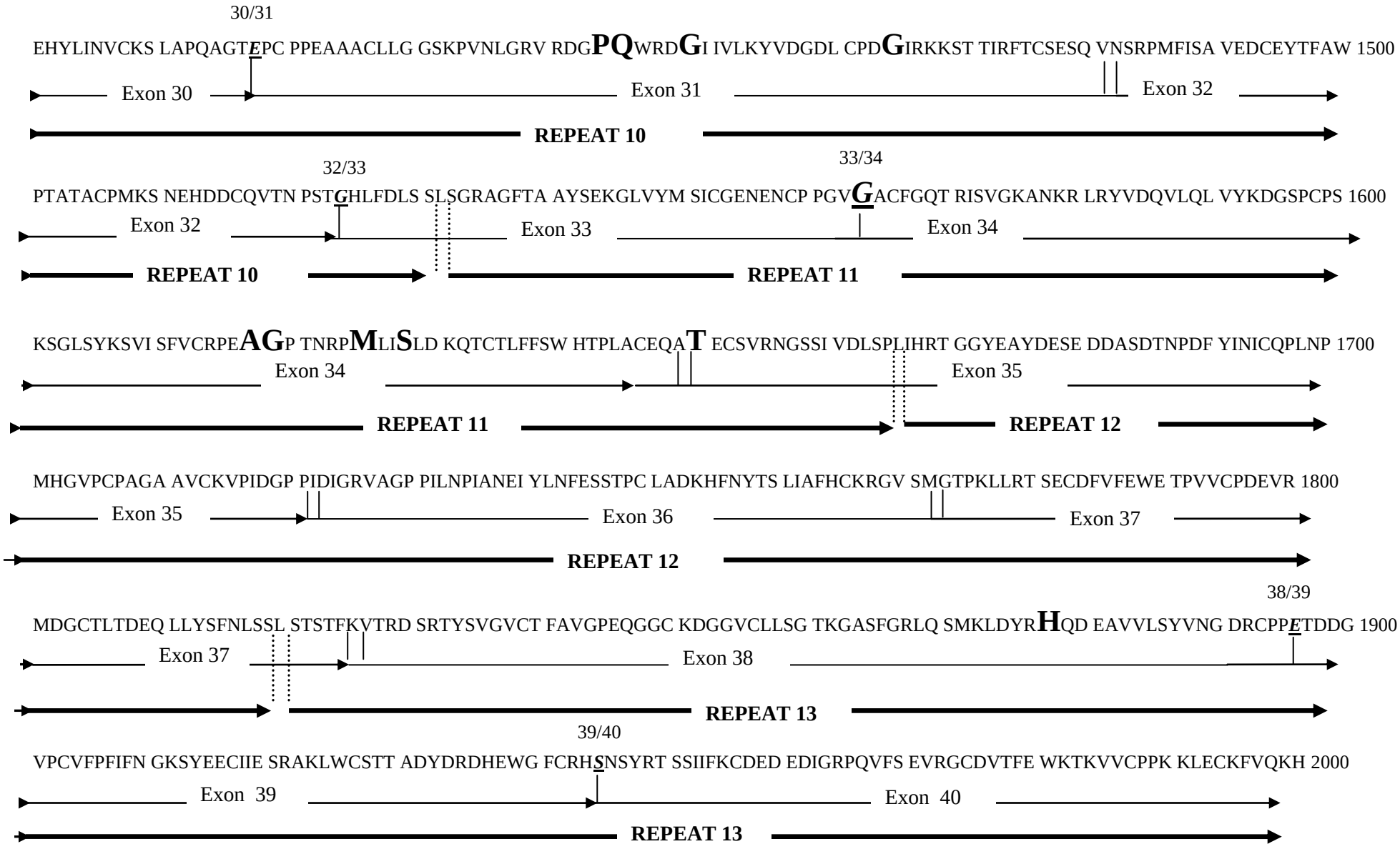
Exon 16
Exon 17
REPEAT 5
GEHVTWRKYY INVCRPLNPV PGCNRYASAC QMKYEKDQGS FTEVVSISNL GMAKTGPVVE DSGSLLLEYV NGSACTTSDG RQTTYTTRIH LVCSRGRLNS 900
Exon 18
Exon 19
REPEAT 6
HPIFSLNWEC VVSFLWNTEA ACPIQTTTDT DQACSIRDPN SGFVFNLNPL NSSQGYNVSG IGKIFMFNVC GTMPVCGTIL GKPASGCEAE TQTEELKNWK 1000
Exon 20
Exon 21
Exon 22
REPEAT 6
REPEAT 7
22/23
23/24
PARPVGIEKS LQLSTEGFIT LTYKGPLSAK GTADAFIVRF VCNDDVYSGP LKFLHQDIDS GQGIRNTYFE FETALACVPS PVDCQVTDLA GNEYDLTGLS 1100
Exon 22
Exon 23
REPEAT 7
24/25
TVRKPWTAVD TSVDGRKRTF YLSVCNPLPY IPGCQGSAVG SCLVSEGNSW NLGVVQMSPQ AAANGSLSIM YVNGDKCGNQ RFSTRITFEC AQISGSPAFQ 1200
Exon 24
Exon 25
REPEAT 8
26/27
27/28
LQDGCEYVFI WRTVEACPVV RVEGDNCEVK DPRHGNLYDL KPLGLNDTIV SAGEYTYYFR VCGKLSSDVC PTSDKSKVVS SCQEKREPQG FHKVAGLLTQ 1300
Exon 26
Exon 27
REPEAT 8
REPEAT 9
28/29
KLTYENGLLK MNFTGGDTCH KVYQRSTAIF FYCDRGTQRP VFLKETSDCS YLFEWRTQYA CPPFDLTECS FKDGAGNSFD LSSLSRYSDN WEAITGTGDP 1400
Exon 28
Exon 29
Poly(G)R insertions and deletions →STOP
REPEAT 9


30/31
EHYLINVCKS LAPQAGTEPC PPEAAACLLG GSKPVNLGRV RDGPQWRDGI IVLKYVDGDL CPDGIRKKST TIRFTCSESQ VNSRPMFISA VEDCEYTFAW 1500
Exon 30
Exon 31
Exon 32
REPEAT 10
32/33
33/34
PTATACPMKS NEHDDCQVTN PSTGHLFDLS SLSGRAGFTA AYSEKGLVYM SICGENENCP PGVGACFGQT RISVGKANKR LRYVDQVLQL VYKDGSPCPS 1600
Exon 32
Exon 33
Exon 34
REPEAT 10
REPEAT 11
KSGLSYKSVI SFVCRPEAGGP TNRPMLISLD KQTCTLFFSW HTPLACEQAT ECSVRNGSSI VDLSPLIHRT GGYEAYDESE DDASDTNPDF YINICQPLNP 1700
Exon 34
Exon 35
REPEAT 11
REPEAT 12
MHGVPCPAGA AVCKVPIDGP PIDIGRVAGP PILNPIANEI YLNFESSTPC LADKHFNYTS LIAFHCKRGV SMGTPKLLRT SECDFVFEWE TPVVCPDEVR 1800
Exon 35
Exon 36
Exon 37
REPEAT 12
38/39
MDGCTLTDEQ LLYSFNLSSL STSTFKVTRD SRTYSVGVCT FAVGPEQGGC KDGGVCLLSG TKGASFGRLQ SMKLDYRHQD EAVVLSYVNG DRCPPETDDG 1900
Exon 37
Exon 38
REPEAT 13
39/40
VPCVFPFIFN GKSYEECIIE SRAKLWCSTT ADYDRDHEWG FCRHSNSYRT SSIIFKCDED EDIGRPQVFS EVRGCDVTFE WKTKVVCPPK KLECKFVQKH 2000
Exon 39
Exon 40
REPEAT 13

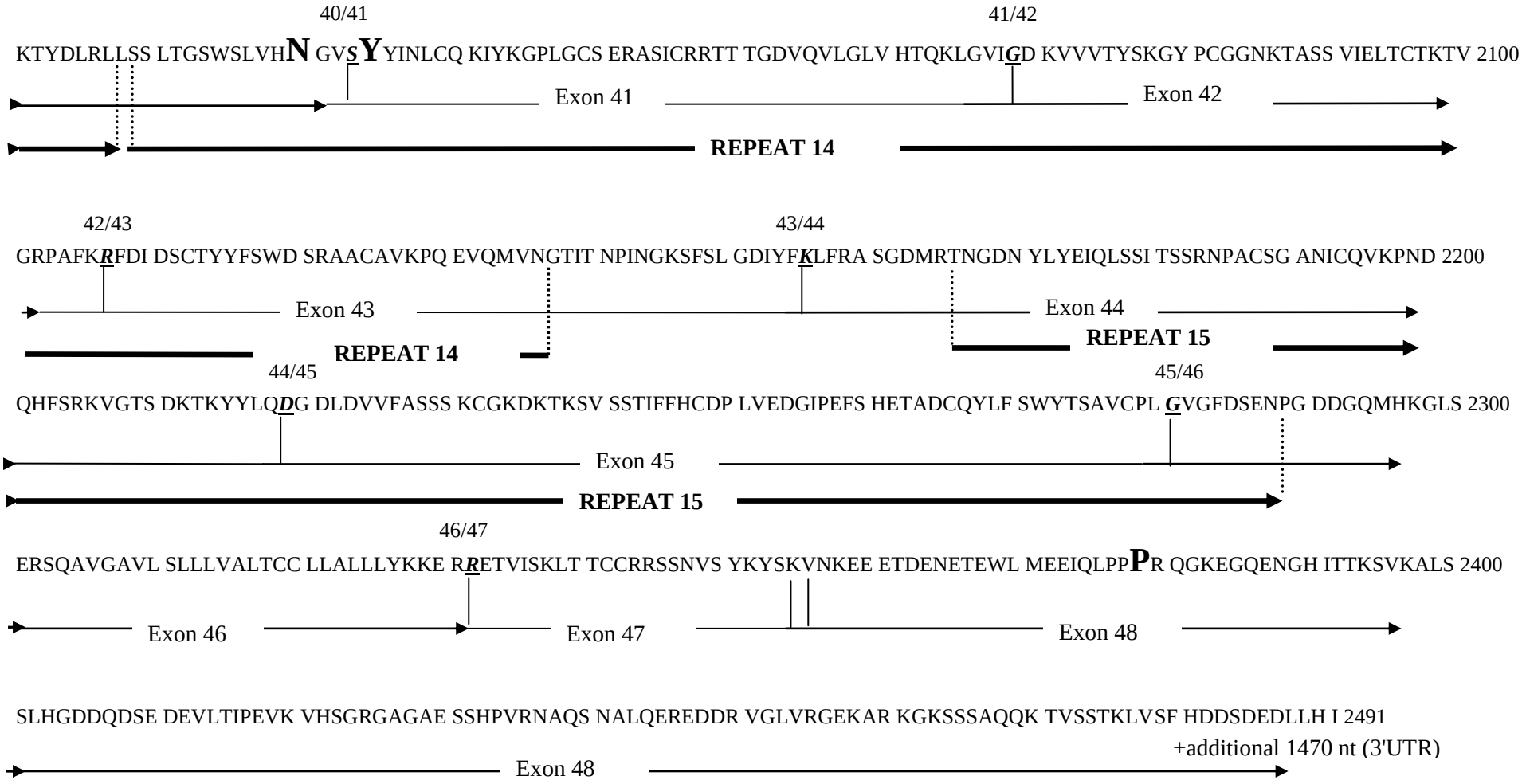


Fig. 1. M6P/IGF2R: coding region and corresponding protein sequence. The letters correspond to amino acids. Italic and underlined: amino acids coded by two exons (numbers of coding exons are given above them). Bold and enlarged letters: positions of mutations (according to the text and Table 1).

Table 1. M6P/IGF2R mutations in human clinical samples

	Total number of samples (patients)/informative number of samples/patients/**LOH**	Mutation Analyses				Reference
		Samples with mutation	Analyzed exons	Mutation found (mutation frequency)	Mutation position	
BREAST	62/40/**12** Informative samples: 21 invasive, 19 *in situ* LOH: 7 invasive and 5 ductal *in situ* (*DCIS*)	2/5	31, 48	Gln1445His (1) Pro2379Thr (1)	Exon 31 Exon 48	[33]
	62/43/**4** Informative samples: 25 early invasive, 18 *DCIS* LOH: 4 *DCIS*	NOT DONE				[34]
LIVER	37(27)/26(18)/**16**(13) informative samples: 18 HCC, 8 dysplastic lesions (DL) LOH: 11 HCC, 5DL; only HCC samples were further sequenced	6/11	27-28, 31	poly($G)_8$, delG (3) Cys1262Ser (1)* Gly1449Val (2)	Exon 28 Exon 27 Exon 31	[43]
	36/22/**14** Informative samples: 16 HCC, 3 fibrolamellar tumors, 3 adenomas LOH: 11 HCC, 2 adenomas, 1 fibrolamellar tumor	5/>11	Whole cDNA	Tyr2024Stop (splice site mutation) (2) Gly1449Val (2) Gly1464Glu (1)	Intron 40 Exon 31 Exon 31	[37,41]
	30/22/**11**	NOT DONE				[42]
	129 (93)/68(54)/**46** Informative samples: 64HCC, 4DLs LOH: 43 HCC, 3DLs	9/46	8-10, 27-35, 37-40	Asp1268Asn (1) Met1625Leu (1) Pro1444Ser (1) Ser1628Phe (1) Gly1315Gln (1) His1878Gln (1) Thr1650Ile (1) poly($G)_8$, insG (2)	Exon 27 Exon 34 Exon 31 Exon 34 Exon 28 Exon 38 Exon 35 Exon 28	[39]
	41/3/**2**	NOT DONE				[44]
	48(42)/27/**1** Informative samples:22 HCC, 2 adenomatous hyperplasia, 3 regenerative nodules LOH: 1 HCC	0/48	27, 28, 31	NOT DETECTED		[38]
PROSTATE	43/36/**15** PIN in 11 out of 15 samples with LOH in tumor. LOH in PIN 7/11	NOT DONE				[45]

HEAD AND NECK	87/56/**30** Informative samples: 17 base of tongue, 11 tonsil, 12 larynx, 9 hypopharynx , 2 paranasal sinuses, 5 oral cavity LOH: 7 base of tongue, 7 tonsil, 9 larynx, 3 hypopharynx, 4 oral cavity	NOT DONE				[52]
LARYNX	35/25/**3**	NOT DONE				[49]
LUNG	22/19/**<u>11</u>**	6/11	8-11, 27-29, 31, 33-34, 37-39	**Gly1296Arg (1)#** poly(G)$_8$, insG (1) **Gly1564Arg (1)#** Ala1618Thr (1) Gly1619Arg (2)	Exon 27 Exon 28 Exon 34 Exon 34 Exon 34	[46]
OVARIUM	4/4/**3**	NOT DONE				[54]
	8/8/**6**	0/6	whole *M6P/IGF2R* cDNA	NOT DETECTED		[51]
ADRENAL GLAND	76/57/**15** Informative adrenocortical tumors: 22 benign, 16 suspect, 19 malignant LOH: 2 benign, 2 suspect, 11 malignant	NOT DONE				[50]
ENDOMETRIUM	46/32/**16**	8/16	27, 28, 33, 34	poly(G)$_8$, insG (2) The rest of mutations not specified.	Exon 28	[53]

Bold underlined: LOH samples that were further sequenced; bold#: possible splice-site mutation.

* mentioned inRref. [55] as “unpublished data”

Bold underlined: LOH samples that were further sequenced; Bold#: possible splice-site mutation

Table 1. M6P/IGF2R mutations in malignant tumors with microsatellite instability (MI)

Clinical cancer samples	Total number of samples	MI-negative	MI-positive	PolyG(8) mutation presence	References
Breast	20	20	0	0	[60]
Endometrium	100	74	26	4	[60]
Ovarium	39	39	0	0	[60]
Esophagus	1	0	1	0	[59]
Pancreas	51	44	7	0	[60]
	2	0	2	0	[59]
Stomach	81	69	12	3	[60]
	29	0	29	7	[59]
Colorectum	114	96	18	1	[60]
	342	279	63	14	[61]
	51	0	51	5	[59]
	90	0	90	23	[62]
	92	0	92	9	[63]
Total number of samples	**1012**	**621**	**391**	**66**	
Mutation presence		0/528	**66/391 (16.8%)**	**← Mutations were present only in MI-positive tumor samples**	
Cancer cell lines and xenographts	**Total number of samples**				
Breast	11 (cell lines)	9	2	2 (MI-positive CAL-51 and MT-3)	[58]
Prostate	28 (6 cell lines, 22 xenografts)	22	6	2 (MI-positive xenograft LAPC4 and MI-negative xenograft PC82)	[64]