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Increased levels of mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2-R) found in serum of patients with breast cancer

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The multifunctional transmembrane mannose-6-phosphate/insulin-like growth factor-2 receptor (M6P/IGF2-R) has been proposed as a putative prognostic marker in breast cancer. Loss of heterozygosity (LOH) with mutations in the remaining allele have been described at the gene level. We previously reported significant decrease of M6P/IGF2-R at the protein level in cancer cells than normal peritumoral ducts of breast tumors which may indicate an increased release of the receptor from the cell membrane to the blood. To assess that hypothesis we measured in this study M6P/IGF2-R levels in serum of women with breast cancer by competitive enzyme-linked immunosorbent assay (ELISA) that allows a detectable sensitivity of 300 ng/mL. The results were obtained referring to a standard curve consisting in varying concentrations of human purified M6P/IGF2-R. Women sera consisted of cancer-free sera (n=39) or serum from women with breast cancer (n=39) were preincubated with anti-M6P/IGF2-R immunoglobulin Y (IgY) antibodies. High levels of M6P/IGF2-R ($p<0.01$) were found in serum from women with breast cancer in comparison with cancer-free serum. Overall the results support the hypothesis of increased release of the receptor from cancer cells to the blood and add one more argument for M6P/IGF2-R studying as a potential breast cancer marker.

Key words: Mannose-6-Phosphate/Insulin-like Growth Factor-2 Receptor (M6P/IGF2-R), loss of heterozygosity (LOH), suppressor gene, breast cancer, competitive enzyme-linked immunosorbent assay (ELISA), IgY, biological marker.

INTRODUCTION

The mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2-R) is a single chain transmembrane protein of 250 kDa that functions as a multifunctional receptor binding many molecules, particularly both molecules sharing the carbohydrates M6P signals and IGF2 (Morgan et al., 1987; MacDonald et al., 1988). It

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contains 2,451 aminoacids with a large extracellular domain made of 2,264 residues, a transmembrane domain of 23 residues and a short cytoplasmic domain of 164 residues. The extracellular domain of M6P/IGF2-R is made of 15 conserved repeat sequences also called homologous domains with an average length of 150 residues and 13% to 37% aminoacids identity which makes it highly conserved throughout species (60% to 90% homology). The M6P/IGF2-R targets newly synthesized mannose-6-phosphorylated lysosomal enzymes such as cathepsins D directly following post-translational modifications from the trans-Golgi network to lysosomes. However, studies reported that pro-cathepsin D is secreted from human breast cancer cell lines suggesting a possible alteration of M6P/IGF2-R (Westley and Rochefort, 1980) The role of cathepsin D in breast cancer has been investigated and results showed that its overexpression and secretion is an independent tissue marker associated with high risk of metastasis in breast cancer (Spyratos et al., 1989; Rochefort, 1992). Moreover, high levels of cathepsin D and pro-cathepsin D have been also described in serum of women with breast tumors and suggested cathepsin D as a poor prognostic marker for metastatic mammary tumors (Brouillet et al., 1997). Others roles of M6P/IGF2-R include neutralizing the mitogenic IGF2 by transporting it to lysosomes for its degradation, activating the Transforming Growth Factor $\beta1$ (TGF$\beta1$) a potent growth inhibitor for most cell types (Dennis and Rifkin, 1991) binding to the retinoic acid (Kang et al., 1998) and targeting the urokinase receptor to the lysosomes (Nykjaer et al., 1998).

To assess the hypothesis of its possible alteration in human breast carcinoma, the M6P/IGF2-R has been studied at the gene and protein levels. The M6P/IGF2-R gene maps to chromosome 6q25-27 (Laureys et al., 1988). Frequent losses of heterozygosity (LOH) at 6q site and mutations in the remaining allele have been observed in human cancer such as hepatocarcinoma (De Souza et al., 1995) mammary cancers (Chapell et al., 1997; Hanskins et al., 1996) and ovarian cancers. (Saïto et al., 1996). Therefore, M6P/IGF2-R gene has been proposed to function as tumor suppressor (De Souza et al., 1995; Oates et al., 1998; Lemamy et al., 2008). However, further studies performed at the chromosome 6q region have not shown any direct relation between gene mutations and tumorigenicity. So, no association was found between M6P/IGF2-R missense single-nucleotide polymorphisms experiments performed on breast cancer multiethnic cohort study and breast cancer risk (Cheng et al., 2009). Likewise, M6P/IGF2-R silencing alone was not sufficient to confer a tumorigenic phenotype but could enhance tumorigenicity in already cancer cells (Caixeiro et al., 2013). However, according to loss of heterozygosity that occurs in M6P/IGF2-R gene in breast cancer, it could be expected to have low protein levels. So, using high-affinity chicken IgY antibodies (M6P/IGF2-R is highly conserved and it has been difficult to obtain high-affinity antibodies in mammals against that protein) we previously showed that M6P/IGF2-R levels quantified by quantitative immunohistochemistry were significantly lower in cancer cells than normal peritumoral ducts of human breast tumors associated with lymph-node invasiveness, which would be consistent with tumor suppressor gene hypothesis (Lemamy et al., 1999).

Interestingly, we showed that in breast carcinomas the M6P/IGF2-R levels were independent of estrogens and progesterone receptors levels, of tumor size and histological grade (Lemamy et al., 1999) indicating that this protein could add any potential value to the existing biological prognostic markers. Furthermore, Confort et al. (1995) reported that the M6P/IGF2-R is secreted from breast cancer cell lines to the culture medium. Further studies showed that M6P/IGF2-R truncation mutants that occur in cancer cells may interfere with function of the normal receptor by dimerization leading to extracellular domain cleavage (Kreiling et al., 2012). The M6P/IGF2-R has also been found in rat serum (Kiess et al., 1987; MacDonald et al., 1989) or in human blood as a circulating protein (Causin et al., 1988) and its role as a putative prognostic marker in breast cancers has been suggested (Lemamy et al., 1999). Subsequent studies showed that the serum form of M6P/IGF2-R is truncated or altered in its cytoplasmic domain with a molecular weight of less than 230 kDa (MacDonald et al., 1989). Therefore, among numerous hypotheses such as a loss of gene expression resulting from a non-sense, frame-shift mutation, loss of heterozygosity in the 6q locus frequently described in human cancers or the receptor shedding from cell membrane, the low levels of M6P/IGF2-R observed in breast cancer cell lines or breast cancer tissues may indicate an increased release of the receptor from the cancer cells membrane to the blood. Consequently, increased levels of M6P/IGF2-R may be expected in the serum of patients with breast carcinoma. In the aim to determine whether such a hypothesis is supported by data we measured circulating M6P/IGF2-R levels in the sera of women with breast carcinoma versus cancer-free sera using competitive ELISA.

MATERIALS AND METHODS

Human tissues and sera origin

Placenta was obtained from the Department of Gynecology. Human normal sera were from cancer-free women (n=39) undergoing hormonal investigation and breast cancer sera were from sera bank of *Laboratoire de Biologie Cellulaire et Hormonale (Hôpital Arnaud de Villeneuve, Montpellier France)* from patients with clinical metastases diagnosed before blood collection (n=39).

M6P/IGF2-R purification

M6P/IGF2-R was purified on a phosphomannan-sepharose affinity column as previously described (Sahagian et al., 1983) with adapted modifications (Lemamy et al., 1999). Briefly, a buffered

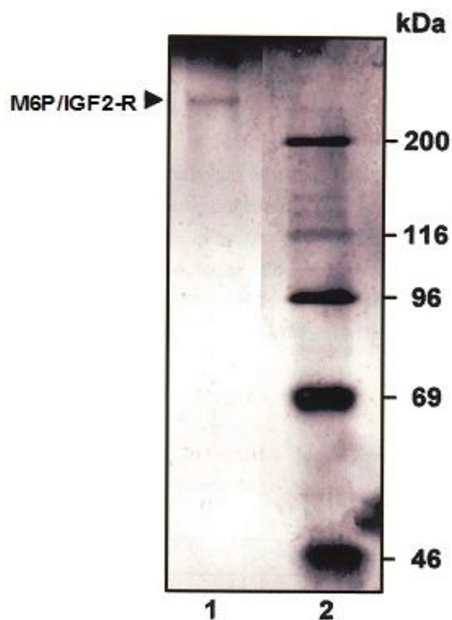

Figure 1. Human receptor purification. Purified human M6P/IGF2-R was revealed by silver staining after migration on a 10% SDS-PAGE as a single band of a protein of approximately 250 kDa (lane 1). Lane 2: molecular weight markers.

extract from acetone powder of human placenta was applied on phosphomannan-substituted affinity column. The M6P/IGF2-R was eluted with 5 mM mannose-6-phosphate and concentrated by lyophilization. The protein assay was performed by Bradford technique (Bradford, 1976) and the purified M6P/IGF2-R was revealed by a 10% SDS-PAGE after silver staining.

Western blot

Western blot was performed as previously described (Lemamy et al., 1999). Briefly, purified human M6P/IGF2-R or 1:2 diluted serum samples were applied to a 8% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad, Ivry-Sur-Seine, France). They were then incubated overnight with IgY antibodies directed against M6P/IGF2-R. After washing, the blots were incubated with rabbit anti-chicken IgY peroxidase-conjugated (Jackson Immuno-Research, West Grove, PA). The bands were revealed using the western-blot chemiluminescence system kit (NEN, Le Blanc Mesnil, France).

Competitive ELISA

ELISA was performed using anti-M6P/IGF2-R IgY antibodies as previously described (Lemamy et al., 1999). Briefly, ninety-six-well ELISA plates Nunc-Maxi Sorp (Poly-Labo, Strasbourg, France) were coated overnight at room temperature with 0.5 µg of purified M6P/IGF2-R diluted in 0.05 M $NaCO_3$/$NaHCO_3$, pH = 9.6. The free sites were then blocked with PBS containing 3% non-fat dried milk (Bio-Rad Laboratories, Ivry-sur-Seine, France) for 1 h 30 min at +37°C.

Standard consisted of varying concentrations of purified human M6P/IGF2-R (from 0.43 ng/mL to 450 µg/mL) preincubated with anti-M6P/IGF2-R IgY (1:800 dilution) in PBS containing 1% non-fat dry milk for 1 h 30 min at +37°C. Women sera, consisted of healthy women serum (positive controls) or serum from women with breast cancer, were diluted from 1:2 to 1:4 and preincubated with IgY antibodies as above. Then 100 µL of either standards or serum samples were added to each well and incubated for 2 h at +37°C and then washed. Peroxidase-conjugated rabbit anti-IgY antibody (Jackson ImmunoResearch Laboratories, USA) diluted in PBS/1% non-fat dried milk (1:2,000 dilution) was added and incubated for 1 h at 37°C. Following washes, the o-phenylenediamine dihydrochloride substrate (CIS Bio international, Gif sur Yvette, France) was added and incubated for 30 min. Absorbance was determined at 492 nm using a multiscan photometer (Titertek Flow, Puteaux, France). Healthy women sera depleted of M6P/IGF2-R by application on phosphomannan-substituted affinity column were used as negative controls.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism version 6.02. Values are shown as mean ± SEM. The differences between groups were assessed using unpaired t-test. The p-value significance was set at 0.05.

RESULTS

Human M6P/IGF2-R purification

The human purified M6P/IGF2-R was revealed by silver staining after migration on SDS-PAGE as a single band of a protein of approximately 250 kDa (Figure 1).

Western blot

The specific IgY antibody (lanes 1 and 2) revealed the M6P/IGF2-R. The purified human receptor appeared as a single band of 250 kDa (lane 1) and the soluble receptor in 1:2 diluted serum (lane 2) was revealed as a major band of approximately 220 kDa. The non-specific IgY (lane 3) did not recognize any component migrating in the 200 to 250 kDa region (Figure 2).

Competitive enzyme-linked immunosorbent assay of circulating M6P/IGF2-R

The least detectable concentration was 300 ng/mL of purified M6P/IGF2-R. Within-run assays performed on a pool of human normal sera gave M6P/IGF2-R levels varying from 3.28 to 4.4 µg/mL with a mean of 3.68 µg/mL and a variation coefficient of 14.5%. In breast cancer patients samples, the M6P/IGF2-R values varied from 3.4 to 6.4 µg/mL, with a mean value of 4.8 µg/mL. Significant increased levels of M6P/IGF2-R ($p < 0.01$) were found in 15 out of 39 sera from women with breast cancer (Figure 3). No signal was detectable with human serum depleted of M6P/IGF2-R by affinity chromatography.

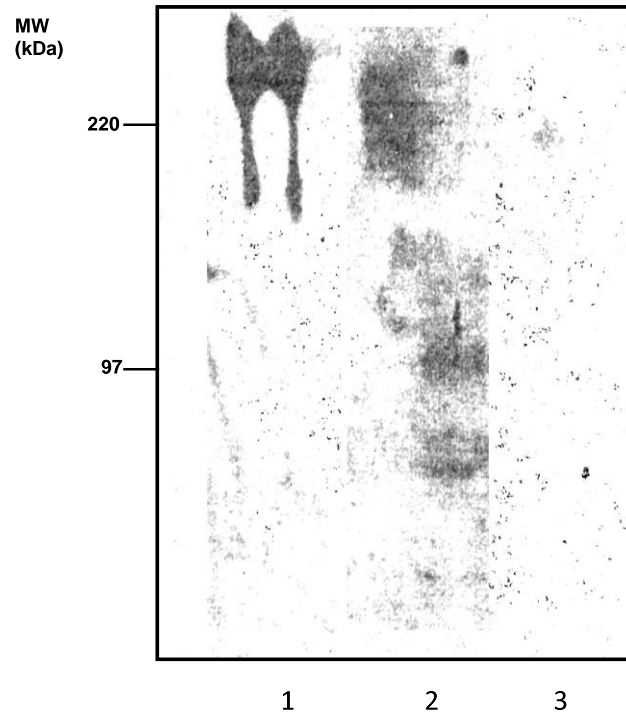

Figure 2. Western blot. Samples (human purified M6P/IGF2-R or serum) were electrophoretically transferred onto nitrocellulose membrane. The purified receptor (lane 1) or serum sample (lane 2) were incubated with specific anti-receptor IgY antibody. The lane 3 shows the non-specific IgY antibody incubated with the purified receptor. The blots were revealed as described in 'Materials and Methods'.

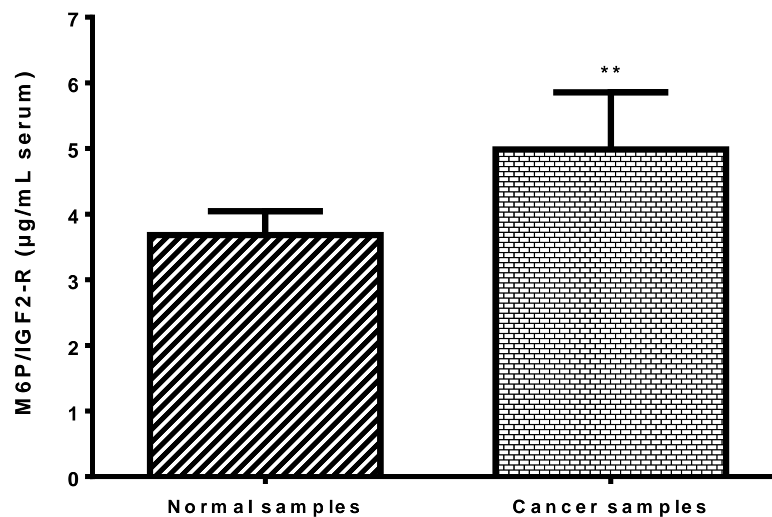

Figure 3. Serum M6P/IGF2-R levels measured by competitive ELISA. Sera samples (normal or from patients with metastatic breast cancer, dilution 1:2 to 1:4) were preincubated with a specific anti-receptor IgY (1:800 dilution) for 1h 30 min. at +37°C, then 100 µL of samples were added to 0.5 µg of purified receptor coated on the solid phase. The presence of IgY on the solid phase was revealed by a rabbit-anti-IgY peroxidase conjugate using OPD as substrate. The results were obtained referring to a standard curve. The M6P/IGF2-R was significantly increased ($p < 0.01$) in serum from breast cancer.

DISCUSSION

The results from competitive ELISA using purified hen IgY anti-human M6P/IGF2-R antibodies previously characterized (Lemamy et al., 1999) showed significant increased levels of Mannose-6-Phosphate/Insulin-like Growth Factor 2 Receptor in 15 out of 39 sera of women with breast cancer in comparison with normal serum ($p<0.01$). There was no detectable signal when human sera were depleted of M6P/IGF2-R by affinity chromatography, showing the specificity of this assay. We previously showed by quantitative immuno-histochemical analysis that the M6P/IGF2-R levels were significantly lower in cancer cells than in normal cells in 10 out of 21 tumors in which the peritumoral normal glands could be quantified in parallel (Lemamy et al., 1999). These results suggested many hypotheses including a loss of gene expression resulting from gene mutation, loss of heterozygosity or receptor decrease due to its release from cell membrane to the blood. Consequently, increased levels of M6P/IGF2-R may be expected in the blood as resulting from its release. Indeed, previous studies reported that the M6P/IGF2-R has been shown to be shed from the cell surface and secreted in the culture medium in MCF7 cancer cell lines (Confort et al., 1995). Moreover, others studies described the M6P/IGF2-R as a circulating protein in rat (Kiess et al., 1987; MacDonald et al., 1989) or in human serum (Causin et al., 1988). According to such hypotheses, the increased levels of M6P/IGF2-R in the sera of women with breast carcinoma observed in the present study could be suggested as resulting from M6P/IGF2-R release from cancer cells membrane to the blood. In a previous study performed in breast cancer tissues, the M6P/IGF2-R levels were found to be independent of estrogen and progesterone receptors levels, of tumor size and histological grade, so the M6P/IGF2-R has been proposed as being a putative prognostic marker in breast cancers (Lemamy et al., 1999). The IgY antibodies used in our ELISA specifically bound to purified and circulating M6P/IGF2-R as shown by the western blot analysis (Lemamy et al., 1999) and the soluble receptor migrated at the 220 kDa region, as previously described (Kiess et al., 1987). The ELISA method has been previously used for the measurement of M6P/IGF2-R in human normal serum (Costello et al., 1999) or for biological markers assay such as plasminogen activators in breast cancers (Grebenschikov et al., 1997; Grebenschikov et al., 1999). Even though the competitive ELISA used in our study may not be enough sensitive for detection since it needed increased amounts of M6P/IGF2-R, it is nevertheless specific for circulating M6P/IGF2-R assay. Furthermore, more sensitive antibodies against M6P/IGF2-R are proved difficult to obtain since this protein is highly conserved throughout mammal species (Lemamy et al., 1999). Therefore, using chicken IgY antibodies based on their high affinity to bind highly conserved proteins in mammals (Stuart et al., 1988) we used a competitive ELISA to measure circulating levels of M6P/IGF2-R in serum of women with breast cancer. High levels of M6P/IGF2-R have been detected in serum of women with breast cancer. These results agree with the hypothesis expecting high levels of this protein in sera of patients with breast cancer and support the hypothesis of increased release of the receptor from cancer cells to the blood and state an additional argument on M6P/IGF2-R studying to determine its potential prognostic significance in breast cancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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