The family of S6K includes an insulin/mitogen-activated serine/threonine protein kinases (S6K1 and S6K2), whose major known substrate is the 40S ribosomal subunits protein S6 [1–3]. S6 phosphorylation is a conserved mitogenic response that regulates translation of 5'-terminal oligopyrimidine tract-containing mRNAs encoding components of protein biosynthetic machinery namely ribosomal proteins and elongation factors [4–5]. Each form of S6K represented in the cell by two isoforms — cytoplasmic (S6K1/II, S6K2/II) and nuclear (S6K1/I, S6K2/I) are known to be generated from a single gene by alternative mRNA splicing and the use of alternative translation start sites [6]. The N-terminal extension in S6K1 sequence contains a nuclear localization signal that targets this isoform to the nucleus, whereas S6K2 appears to be localized predominantly in the cytoplasm [7].

At least two distinct signaling pathways underlying activation of S6K1 have been identified. One pathway involves PI 3-kinase, and its downstream effectors PDK1 and perhaps PKB [8]. Treatment of cells with PI 3-kinase inhibitors such as wortmanin inhibits the activation of S6K. Another pathway contributing to the activation of S6K is regulated by the mammalian target of rapamicine (mTOR) as revealed using the immunosuppressive rapamicine, which inhibits mTOR and activation of S6K [9].

S6K1 was identified more then decade ago, and S6K2 was identified only recently, hence most functional studies have involved S6K1 [10–13]. S6K2 phosphorylates S6 ribosomal protein, and is highly homologous to S6K1 in the core kinase and linker regulatory domains [10–13]. Seven of eight mitogen-stimulated regulatory phosphorylation sites identified in S6K1 are conserved in S6K2. These suggest that S6K2 may be regulated through the multisite phosphorylation mechanism that is similar to that for S6K1. At the same time there are interesting differences in S6K2 primary structure that may confer differential regulation and functioning of this kinase [10]. C-terminus of S6K2 contains a proline–rich domain, which is thought to be involved in a specific interaction with regulatory proteins [10, 14], and additional nuclear localization signal not found in S6K1 [12].

Taken together, these findings raise the possibility that S6K2 is regulated by a similar but distinct mechanism from that for S6K1.

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Taken together, these findings raise the possibility that S6K2 is regulated by a similar but distinct mechanism from that for S6K1.
There is a growing evidence that S6K kinases exhibit oncogenic potential. The gene encoding S6K1 has been found to be amplified and overexpressed in the MCF-7 breast cancer cell lines and in approximately 10% of human breast carcinomas which is associated with poor prognosis [15–17]. Some authors believe that S6K1 is the first oncogenic marker that has prognostic impact on locoregional control and therefore may have clinical implication in determining the local treatment strategy in early-stage breast cancer patients [17]. We have also reported overexpression of S6K1 kinase in breast adenomas and adenocarcinomas [18], thyroid [19] and endometrial tumors [20]. Constitutive activation of S6K1 was shown in primary human thyroid adenomas and myeloma [21], increased expression of phosphorylated S6K1 was detected in papillary thyroid tissues [22]. So far very little is known about oncogenic potential of S6K2, which has been discovered much later than S6K1. According to our previously published data S6K2 is overexpressed in small cell lung cancer cell lines [23], breast tumors and breast cancer cell lines [19]. Recently we have performed immunohistochemical analysis of S6K expression in benign and malignant breast tumors [24]. Together with overall increase in the amount of S6K1 and S6K2 in cancer cells a strong accumulation of S6K2 kinase in the nuclei of epithelial adenocarcinoma cells have been detected in several tumor samples in contrast to the normal tissues. This fact has not been described in the literature before and demonstrates divers functioning of S6K1 and S6K2 during oncogenesis. In present study we performed a comparative immunohistochemical analysis of subcellular localization of S6K kinases.

**MATERIALS AND METHODS**

Tissue samples of the breast carcinomas were obtained from the collection of Center for Immunohistochemical Diagnostics (Dnipropetrovsk, Ukraine). All samples were classified according to the criteria proposed by WHO. From 150 cases 58 were randomly chosen for investigation. All tumors were primary without any treatment. Infiltrating ductal adenocarcinomas in 42 cases and infiltrating lobular adenocarcinomas in 16 cases were diagnosed. From them 5 had I, 26 — II, 11 — III histologic tumor grade, 9 — contained mixed differentiated tumor structures. For comparison of localization of investigated antigens 5 conventionally normal breast tissue samples and 4 breast adenomas were used. The age of the patients ranged from 27 to 76 years.

All tissue samples were fixed in formalin and embedded in paraffin. For analysis we used 5 µm sections after deparaffinization. Immunohistochemical staining of breast tumor tissues have been performed as it was described earlier for thyroid gland tumors [19] and breast tumors [18]. For blocking of nonspecific binding we used Biotin blocking system (DAKO, USA) and 1% BSA (Diam, Russia). The dilution of rabbit polyclonal anti–S6K1 antibodies was 1 : 5000 and anti–S6K2 mouse monoclonal antibodies was 1 : 12 000. Anti–mouse and anti–rabbit biotin labeled antibodies (Sigma, USA) and Vectastain ABC Kit (Vecor Lab., USA) were used. For negative control 1% BSA (Diam, Russia) was added instead of primary antibodies. Specificity of primary antibodies has been analyzed by staining of tissue slices using anti–S6K antibody preliminary blocked by recombinant antigen (C–terminal peptide of S6K) as described earlier [24]. No staining has been observed in this case.

Light microscopy was performed using an AxioPlan microscope (Carl Zeiss, Germany). Immunostaining of cancer cells was evaluated and scored for cytoplasmic localization as: 0 — no staining, 1 — weak staining, 2 — moderate staining, 3 — strong staining; for nuclear staining: 0 — no staining, 1 — 1–20% positively stained nuclei, 2 — 20%–50% positively stained nuclei, 3 — more then 50% positively stained nuclei. If staining was greater or equal than two, staining was deemed as overexpression and tumor positive for S6K1 or S6K2 [17].

**RESULTS**

Earlier we have studied the profile of S6K1 and S6K2 expression in different human breast tumors by Western blotting analysis of tumor’s homogenates. An elevated level of S6K1 expression has been detected in approximately 60% of benign and malignant tumor samples but expression of S6K2 was elevated only in malignant tumors almost to the same extent as S6K1 [18]. According to immunohistochemical analysis of breast fibroadenomas and adenocarcinoma tumors S6K kinases are overexpressed not only in the cancer cells of epithelial origin but in stroma, vessels and smooth muscle cells [24]. Sometimes the overall amount of S6K1 kinases, especially S6K1, was even greater in non–epithelial cells. However the most interesting finding was the accumulation of both kinases in the nuclei of cancer cells that was more typical for S6K2 and has not been observed in normal tissues. In some cases immunostaining of the nuclei with specific anti–S6K1 or anti–S6K2 was even greater than in non–epithelial ones. We believed that at least for S6K we observed the nuclear accumulation of the cytoplasmic form of p70S6K2 kinase since according to the Western blotting analysis of tumor samples overexpression of S6K2 nuclear 56kDa form (S6K2/l) have not been detected [18]. To investigate these phenomena in more detail we performed extended immunohistochemical analysis of tumor samples and focused only at distribution of S6K kinases between cytoplasm and nuclei in transformed epithelial cells. We have analyzed 5 samples of normal tissues distant from tumor, 4 samples of fibroadenoma and 58 adenocarcinoma tumors.

Normal tissues exhibited very weak staining of the cytoplasm of epithelial cells for both kinases (Fig. 1). Staining of the nuclei has not been detected at all. That was a typical staining of normal breast tissues we have described before [18]. Analysis of benign tumor samples revealed more intense staining of S6K1 and S6K2 in the cytoplasm of tumor cells versus normal tissues and in some cases was estimated as moderate or even strong. The overall increase of S6K1 and S6K2 in the cytoplasm co–
pared to normal tissues was 25% and 50% respectively. Very weak staining of the nuclei has been detected in a half of cases, but only for S6K2 (see Fig. 1). In malignant tumors in 10.0% cases an elevated level of S6K2 in the cytoplasm have been detected. In contrast to normal and benign tissues where nuclear localization have not been detected at all, 22% of cases demonstrated nuclear localization of S6K1, in 8.6% of them up to 50% of nuclei were stained.

The cytoplasm staining of S6K2 in breast adenocarcinoma samples was a little lower then in benign tumors but still grater then in normal tissues — up to 25% of the cases revealed S6K2 overexpression (6.9% of them — the strong one). We have detected a dramatic increase in a number of nuclear positive and decrease in cytoplasm positive cancer cells for tumor grade III that has to be the most aggressive tumor type.

Detailed analysis of localization of S6K1 and S6K2 in tumor cells revealed 4 different variants of kinase distribution (Fig. 3): absent or basal expression level in the cytoplasm and the nuclear staining of less then 20% of cancer cells (86% and 36% of cases respectively); only nuclear staining of more then 20% of cancer cells (5% and 36%); only increased cytoplasmic staining (5% and 10%); simultaneous expression in the cytoplasm and nuclei (3% and 17%). These data indicates that in very limited number of cases S6K1 and S6K2 kinases (especially S6K2) have been detected exclusively in the cytoplasm.
Comparative analysis of p70S6 kinases expression in cancer cells lead to the assumption about compensatory effect in kinase expression. In spite of high number of S6K1 and S6K2 cytoplasm–positive cells (32%, Fig. 4), only in 3% of cases expression of both forms have been detected. In the nuclei co–expression was detectable only in 22% of cases along with 80% overexpression of S6K2. In addition detection of S6K1 in the nuclei (in contrast to S6K2) always was accompanied with S6K2 and S6K1expression.

**Fig. 4** Combination of S6K1 and/or S6K2 distribution in cytoplasm and in nuclei in breast cancer cells

**DISCUSSION**

S6K1 and S6K2 are each represented in the cell by two isoforms encoded by the same gene and originated by use of alternative translation starts. The 23– and 14–aminoacids extension at the N–termini of S6K1/I and S6K2/I contain nuclear localization signals (NLSs) that target these isoforms constitutively to the nucleus [10–12]. Earlier we already claimed an overexpression of both kinases in breast tumors by Western blotting analysis of tumor extracts and in this study we have confirmed our previous data using alternative methodological approach. However, if for S6K1 in some tumor samples we have detected an elevated level of nuclear (85 kDa) isoform along with the cytoplasmic (70 kDa) for S6K2 we detected only overexpression of cytosolic isoform (54 kDa). Overexpression of both isoforms (nuclear and cytosolic) of S6K1 could be explained by amplification of the S6K1 gene detected in cells of adenocarcinoma MCF–7 cell line and in 8.8% of primary breast tumors [15–16]. S6K1 gene was found to be located to 17q23 chromosomal region that is a frequent site of gene amplification in breast cancer. Interestingly that according to the data of the same authors [16], the elevated level of S6K1 protein was found in approximately 16% of cases indicating existence additional mechanisms responsible for the overexpression of S6K1. Amplification of S6K2 gene has not been shown so far but it is necessary to point out that gene encoding S6K2 was mapped to chromosomal region 11q13, which is associated with acute myeloid leukemia, non–Hodgkin’s lymphoma, chronic lymphoproliferative disorders etc. Western blotting analysis of S6K2 expression in breast tumor samples along with the overexpression of cytosolic isoform (54 kDa) does not reveal any changes in the amount of nuclear isoform (60 kDa) suggesting regulation of S6K2 expression on the level of mRNA splicing or translation of correspondent mRNA.

The presented data coincides with literature one and confirms our previous data indicating overexpression of both S6K kinases in breast tumors. However the most interesting finding of this study is accumulation of S6K1 and most probably cytosolic form of S6K2 in the nuclei of cancer cells. For the first time we have demonstrated strong accumulation (up to 80%) of S6K2 in the nuclei of cancer cells. The possible nuclear localization of S6K2/II have been predicted before [12] due to the presence of additional NLS at the C–terminus of both splice variants of S6K2. There is an experimental evidence supporting this idea. Confocal microscopy of CHO–IR cells transfected by S6K2/I–GFP and S6K2/II–GFP constructs demonstrated accumulation S6K2/I in the nuclei and dispersed distribution S6K2/II in the cytoplasm and nucleoplasm [25]. We have observed as well predominantly nuclear localization of S6K2 in HEK cells transfected by construct coding for cytoplasmic form of S6K2 [26]. Treatment of the same cell by Leptomycine B (LMB), agent that blocks nuclear export leads to the complete translocation of S6K2 from the cytoplasm to the nucleus suggesting nuclear shuttling of the cytoplasmic form of S6K2 [26] and retention in the nuclei mediated by LMB. Interestingly that in present study we could detect S6K2 only in the cytoplasm of epithelial cells of normal breast tissues but not in the nuclei suggesting that in nonproliferating cells S6K2/II has to be localized predominantly in the cytoplasm. It is necessary to point out that nuclear shuttling have been demonstrated for the cytoplasmic form of S6K1 as well however it doesn’t have NLS. LMB treatment of the cell led to the accumulation of this kinase in the nuclei as well [26] demonstrating the existence of other then S6K2 mechanisms of nuclear/cytoplasmic shuttling.

Nuclear shuttling has been demonstrated for the one of the main effectors of p70S6 kinases — mTOR [27]. Having no influence on mTOR activity, nuclear shuttling seems to be very important for the functioning mTOR and spatially for the 5’–TOP–dependent mRNA translation initiating. In this context we can predict that nuclear translocation of both forms would be necessary event for the kinases activation and the transducing of mitogenic stimuli in the cell. It is possible that in quiescent cells nuclear shuttling of cytoplasmic S6K2 is strongly regulated by upstream effectors preventing kinase from nuclear translocation and only mitogenic stimuli may promote this process. In this case nuclear translocation may indicate mitogenic activation of kinase. Recently it was demonstrated that PKC may phosphorylate S6K2 (but not S6K1) and cause kinase retention in the cytosol [26]. The phosphorylation site was determined, it is located within C–terminal NLS of S6K2. It is possible that PKC is one of the main regulators of kinase translocation in or out of the nuclei. Though S6K1 has no additional NLS and have not been shown to be a substrate for PKC there is a data about PKC/p70S6x complex formation suggesting idea that PKC may be involved in the regulation of p70S6x translocation as well [28]. Deregulation of PKC functioning during oncogenesis may be a reason of abnormal p70S6 kinases accumulation in the nuclei of cancer cells. Deregulation of nuclear export that has not been studied yet could have a contribution in this process as well.
Activation of p70S6K in response to the mitogenic stimuli may have place right in the nuclei as well as phosphorylation of S6 ribosomal protein that involved in nuclear ribosomal biogenesis. This is supported by the data from G. Thomas laboratory [29]. Studies with transgenic mice of S6K1-/-/S6K2+ and S6K1+/S6K2- genotype have demonstrated that elimination of functional S6K2 in contrast to S6K1 leads to dramatic reduction in the amount of phosphorylated S6 including nuclear form. Summarizing the presented data we can assume that nuclear accumulation of S6K kinases in the nuclei may reflect mitogenic activation of kinases, however for such conclusions an additional studies has to be performed.

The possible targets of S6K in the nuclei of cancer cells could be transcriptional factor CREM [30] and recently identified S6K substrate SCAR protein that involved in mRNA splicing and transport [31]. Unfortunately phosphorylation of mentioned substrates has been demonstrated only in vitro systems.

In this study using immunohistochemical approach we have confirmed the overexpression of S6K in breast tumors [18, 24]. For the first time we demonstrated strong accumulation of S6K2 and to a less extent S6K1 in the nuclei of cancer cells if compare with normal tissues and benign tumors. Taking into account that overexpression of S6K1 has been shown to be correlated with poor prognosis at breast adenocarcinoma and possible functional compensation between S6K1 and S6K2 proposed by other authors as well [16, 17], the further verification of prognostic and diagnostic value of S6K2 should include comparative analysis of patients diagnosis with peculiarities of S6K expression and subcellular localization.

REFERENCES


ИММУНОГИСТОХИМИЧЕСКИЙ АНАЛИЗ ЛОКАЛИЗАЦИИ S6K1 И S6K2 В ОПУХОЛЯХ МОЛОЧНОЙ ЖЕЛЕЗЫ

Цель: провести анализ экспрессии и особенностей локализации S6K в аденоами и аденокарциномах молочной железы человека и в нормальных тканиях молочной железы. Методы: уровень экспрессии и локализацию S6K анализировали в гистологических срезах нормальной ткани молочной железы человека, аденоами и аденокарциномами разной степени дифференцировки, предварительно фиксированных формалином и заключенных в парафин. Иммуноцитохимическую детекцию S6K1 и S6K2 в эпителиальных клетках молочной железы осуществляли с помощью специфических монооксиных и полиоксиных антител к S6K1 и S6K2 с последующим полуколичественным анализом данных. Результаты: обнаружено повышенное содержание S6K в цитоплазме опухолевых клеток эпителиального происхождения доброкачественных и злокачественных опухолей молочной железы по сравнению с эпителиальными клетками нормальной ткани. В аденокарциномах выявлено накопление в ядрах опухолевых клеток S6K1 и в большей степени S6K2. В 80% аденокарцином молочной железы детектировано ядерное окрашивание S6K2 по сравнению с нормальными тканями. В 31% случаев почти в 50% опухолевых клеток наблюдалось сильное окрашивание ядер. Ядерная локализация S6K1 в опухолевых клетках установлена в 25% аденокарцином. В эпителиальных клетках нормальной ткани молочной железы ядерная локализация S6K не обнаружена. Вывод: иммуногистохимический анализ свидетельствует о гиперэкспрессии обоих форм S6K (S6K1 и S6K2) в опухолях молочной железы человека. Полуколичественный анализ особенностей локализации S6K в эпителиоцитах молочной железы свидетельствует о высоком содержании S6K (особенно S6K2) в нуклеоплазме, что является характерной особенностью опухолевых клеток.

Ключевые слова: S6K1, S6K2, иммуногистохимия, аденоами и аденокарциномы молочной железы.