RAB family gene expression in breast cancer cells under influence of paclitaxel

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ABSTRACT

The aim of this study was to investigate the role of paclitaxel on RAB family of genes in primary breast cancer cell lines. The cancer breast cells obtained from 40 women during mastectomy were used to address this issue.

The group included patients with intraductal breast cancer – lesions in I or II advancement level by TNM classification and G1-G2 by Bloom classification. (tumor dimensions up to 2.0 cm without metastases to lymph nodes). Cytostatic drugs before surgery were not administered to these patients.

The cultures were conducted in 25 cm^2 plastic containers at RPMI medium with addiction of 10% fetal bovine serum (FBS) at the standard conditions. After reaching concentration levels of 10 000/ml of the cells, the cultures were treated with 60 ng/ml and 300 ng/ml doses of paclitaxel. The concentrations were calculated in relation to therapeutic doses of paclitaxel, applied in polytherapy in patients with breast cancer. The cell cultures untreated for cytostatic were used as a control group. To these cultures, 5% DMSO was administered.

Analysis was conducted for RAB family of genes: RAB3D, RAB5B, RAB5C, RAB7, RAB7L1, RAB9P1, RAB10. RAB11A, RAB311B, RAB13, RAB18, RAB22A, RAB23, RAB26, RAB27A, RAB27B, RAB28, RAB30, RAB31, RAB33A, RAB3D6, RAB 38, RABL2B

Total RNA was extracted from the harvest control group and the treated cells, and this was followed by cDNA synthesis, which was used for hybridization assays using arrays. A lower dose of paclitaxel (60 ng/ml) treatment resulted in an increase (2-4 fold- statistically significant), whereas a higher dose (300 ng/ml) caused a decrease (2-fold – statistically insignificant) in expression of examined oncogenes, compared to that of the control group.

In summary, this data indicates that 60 ng/ml paclitaxel dose induced the RAB gene expression in an up-regulated pathway. A higher concentration of cytostatic (300 ng/ml) is a toxic dose for primary breast cells in vitro.
Keywords: breast cancer; paclitaxel; RAB family genes; microarray

INTRODUCTION

In the metabolism of neoplasm cells, processes involving division and growth play an important role. It has been reported that alterations in gene expression involved in the cell cycle were also characteristic of an increased activity of genes associated in cell proliferation and growth. These changes usually correlate with a decreased expression of genes that control growth, division and transcription processes in the cells. Oncogenes play a very important role in the transformation of normal cells to neoplastic cells. A significant number of these genes encode signaling proteins within the cell.

Some of these code factors influence cellular growth (sis, int) and growth factor receptors (erb-B, fms). In this group, there are known genes which code transcription factors (myb, myc, jun, fos), kinases (src,yes, abl, raf, mos), GTP binding proteins (ha-ras, ki-ras, n-ras) (1).

Some of these genes cause the transformation from normal cells to neoplastic cells when mutation in their structure occurs.

BRCA1 belongs to the suppressor genes group, which is an activator of the transcription process, involved mainly in the DNA repairing system (2). The genes that belong to this group can inhibit or activate cell growth and proliferation in 2 ways. One of them inhibits transformation processes when they are not damaged by mutations. The normal function of these genes stops cell proliferation. P53 codes protein responsible for phosphorylation products of different genes (3). It plays a crucial role in controlling the cell cycle by activating more than 30 genes (4,5). P-53 activates genes encoding proteins which can inhibit apoptosis. This protein stops the cell cycle in the G1 and G2 phase, which can enable DNA repairing. MDM-2 is the main protein that causes the decrease of p-53 level. MYC protein is responsible for the increased level of p53 and ARF. The active form of P53 can be released from the MDM/P53 complex (1). The higher expression of these genes activates proliferation processes. The mentioned genes (RAB, ACT, MYC, JUN, FOS) can activate faster growth, division of cells and transformation processes.

MATERIALS

The cancer cells were obtained from breast tissues of 40 women with histopathologically confirmed breast cancer. The group included patients with intraductal breast cancer – lesions in I or II advancement level by TNM classification and G1-G2 by Bloom classification. (tumor size up to 2.0 cm without metastases to lymph nodes). There patients were not administered any cytostatic drugs before surgery. The primary cultures were conducted in 25 cm^2 plastic containers at RPMI medium with 10% fetal bovine serum (FBS SIGMA), at standard conditions (37°C, 5% CO_2, 90% humidity of the air).

Paclitaxel in 60 ng/ml and 300 ng/ml doses was administered to these cultures after achieving concentration of the cells 10 000/ml. The cytostatic concentrations were calculated in relation to therapeutic doses of paclitaxel applied in polytherapy in patients with breast cancer. The control culture was a suspension of the breast cancer cells without the administration of cytostatic treatment. To these cultures 5% DMSO was administered. The experiments were conducted in three independent trials for all mentioned groups of cultures.

Analysis was related to RAB family of genes : RAB3D, RAB5B, RAB5C, RAB7, RAB7L1, RAB9P1, RAB10. RAB11A, RAB311B, RAB13, RAB18, RAB22A, RAB23, RAB26, RAB27A, RAB27B, RAB28, RAB30, RAB31, RAB33A, RAB3D6, RAB 38, RABL2B.

METHODS

After an incubation period of 72 hours with two doses of paclitaxel, the cultures were homogenized in TRI (SIGMA) solution. RNA (total RNA) obtained from the cultures was controlled with the presence of impurities and DNA. It was controlled on a spectrophotometer (EPPENDORF).

Panorama Human Cancer cDNA Labeling and Hybrydyzation Kit; CDLBL-HCN (SIGMA-GENOSIS) was used to obtained cDNA from total RNA. The kit included control RNA from E. coli (Panorama Armored RNA E. coli-B 1444 RNA). RNA from E. coli let calibrate expression of examined genes in comparison to this gene.

Water was added to the samples containing 2 µg total RNA (4 µl) and 2 µl control RNA Escherichia coli reaching 10.0 µl. Mix (dATP, dGTP, dTTP) and labeled ^{32}P dCTP of 40 µCi activity , 6 µl reverse transcriptase and solution buffer (4 µl) were added to the samples. This mix was incubated at 42 ° C for 2.5 hours. In reverse transcription, the reaction cDNA containing labeled ^{32}P cytidine was estimated. Such received and purified cDNA was hybridized on the array. The Sigma-Genosis array contained sequences of oncogenes on the ground. cDNA was hybridized with the array in the hybridization chamber at 65° C for a period of 13 hours. After purifying the array and removing unconnected fragments of cDNA

the ground was dried on a sterile blotting and placed in a cassette with a radiosensitive ground to collect the activity from points of the array (Screen Imaging K – Bio Rad) for 24 hours. After radiation of the radiosensitive ground, using a high resolution scanner it was scanned (Molecular Imager FX -BioRad) to estimate the picture of the array. Activities from the subsequent points of the array were normalized with the expression level of E. coli-B genes localized in control points. Statistic analysis was conducted by using program Statistica 6.0.

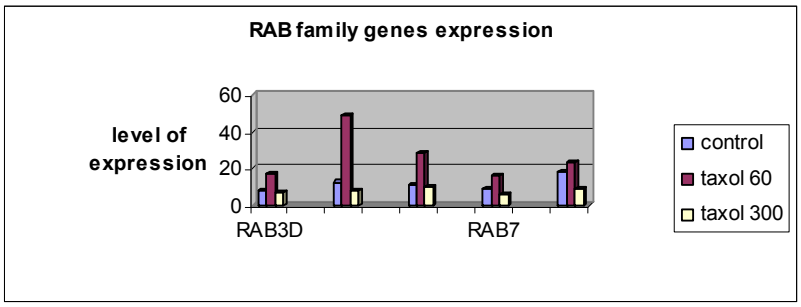
RESULTS

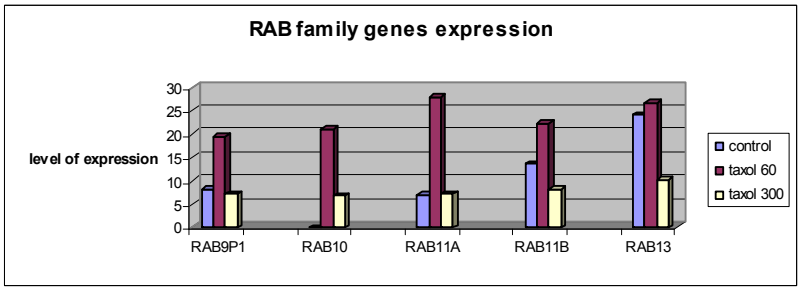
Analysis showed that in a group of cells where paclitaxel was administered at a dose of 60 ng/ml it caused statistically significant increase expression RAB family of genes in comparison to the control group. In the group where paclitaxel was administered at a dose of 300 ng/ml, a statistically significant decrease expression of the genes coding oncogenes was observed in comparison to thecontrol group.

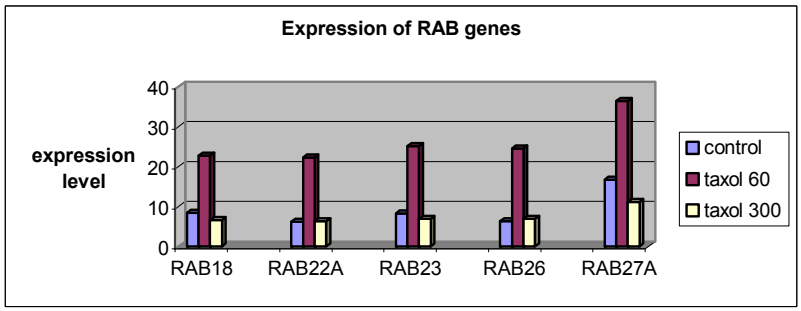
There was a statistically significant correlation between the gene expression of the control group and both mentioned primary breast cell cultures after the administration of paclitaxel ($P<0.001$).

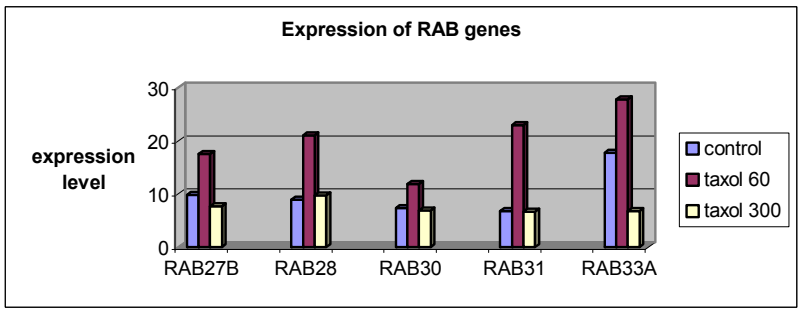
Table 1. Expression of RAB family genes.

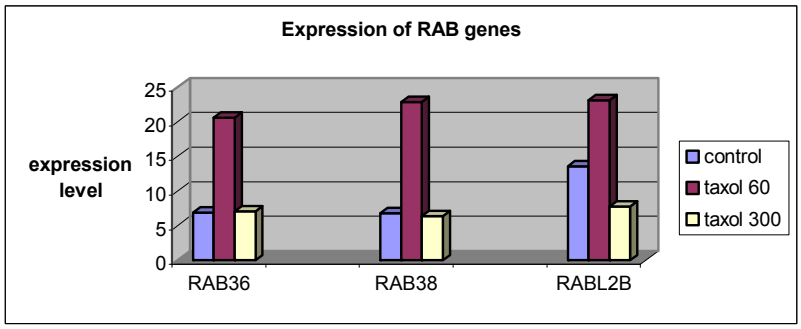
GENES	**CONTROL**	**TAXOL 60**	**TAXOL 300**
RAB3D	8.11	17.43*	6,90
RAB5B	12.67	48.74*	8.11*
RAB5C	11.28	28.39*	10.46
RAB7	8.97	16.14*	5.83*
RAB7L1	18.39	23.13*	8.96*
RAB9P1	8.25	19.50*	7.20
RAB10	8.70	21.10*	6.84
RAB11A	6.90	27.78*	7.19
RAB11B	13.57	22.13*	8.23*
RAB13	23.95	26.45	10.20*
RAB18	8.46	22.60*	6.66
RAB22A	6.27	22.28*	6.42
RAB23	8.26	25.20*	6.94
RAB26	6.36	24.60*	6.97
RAB27A	16.75	36.51*	11.13*
RAB27B	9.87	17.55*	7.76*
RAB28	8.93	21.10*	9.76
RAB30	7.40	11.92*	6.90
RAB31	6.83	22.94*	6.75
RAB33A	17.76	27.85*	6.76*
RAB36	6.89	20.58*	7.08
RAB38	6.85	22.86*	6.34
RABL2B	13.56	23.17*	7.78*











DISCUSSION

Oncogenes are regulators of transcription. They are factors that inhibit apoptosis and create transcription factors. Many of them cooperate with sections of DNA coding regulators of gene and promoters. Products of these genes are responsible for the stimulation of cell growth and proliferation. This group presents such analyzed genes as: ASK, PIM1, RAF, RAB, RAN, ERBB2-4. A different group is responsible for both proliferation and activation of apoptosis. MYC, AP1, TEL belong to such researched genes. In such group of analyzed genes, oncogenes, mainly with apoptosis, such as DAXX. MAX, can surface

Activation of MYC genes caused by paclitaxel in the cells proves that in researched cultures different metabolic pathways can coexistence simultaneously, can stimulate induction and inhibition of proliferation. Many products of different genes cooperate with Myc in growth and transformation and lead to the inhibited apoptotic characteristic of these proteins. Transformation and growth of cancer cells are observed with the increase of expression Bcl-2 and MYC gene and decrease expression of BAX (7). The data indicates that the influence of paclitaxel induces not only growth of the expression of MYC which leads to stimulation., but also antiapoptotic processes which accelerates transformation.

Distinct metabolic activation stimulated by Myc and p53 was observed during this study. Myc activates P-53 with the participation of P19/ARF protein (6). Myc also stimulates these proteins: Bax and Puma. They are connected with apoptosis and activate P-53 (8,9). This data indicates that MYC plays a role as an apoptotic activator. This gene can inhibit the activity of genes encoding the transcriptor activator MIZ-1 through direct connection with them. Myc also regulates processes connected with proliferation (10,11). With regards to the apoptosis activation by paclitaxel we should pay attention to the possibility of the activation of these process by means of the P53 gene. Since the product of this gene is connected with the control phase of G1/S, it should be noted that paclitaxel is influenced by the regulation of the earlier phases of cell cycles. It suggests that sensitivity for paclitaxel is marked not only in G2/M phase, but also in G1/S phase. Perhaps the increase response of paclitaxel is conected to the inhibition of both these cell cycle phases and caused the inhibition cell division in the G1/S phase. On the other hand, in other studies (12) authors did not observe any cross resistance between taxanes and drugs influenced in the G1/S phase. It suggested that paclitaxel does not influence the first phases of the cell cycle. An investigation conducted by Kandioler-Eckersberger (13) showed that a group, of 67 patients with the mutated P53 gene, responsed to treatment with paclitaxel. There was no correlation found between the response to this treatment and the amount of apoptotic cells in patients with normal function of the P53 gene. Significant response to paclitaxel was observed in patients with structural damage of this gene. These investigations indicate that paclitaxel can regulate the cell cycle of G1/S phase and damaged P-53 protein does not influence this process. It seemed that

MYC is a gene responsible both for inhibition of cell cycle and apoptosis. The inhibition of P21, through activation of the MYC/MIZ-1 complex, plays an important role in this process. This process probably plays a key role in switching cell cycle from stopping cell growth to apoptosis (14,15). This indicates that genes responsible for stopping growth and apoptotic activators should be analyzed separately and not joined in the same group activators of programmed cell death.

Proliferation processes are activated by (PI3K) phosphatydyloinositolo-3 kinase and Rab gene products. Growth factor activates PI3K and Rab gene products which increases the activity of PDT/INS(3,4,5)P3 - phosphatydyloinositolo-3,4,5 phosphatase (16) .The aims of PDT/INS(3,4,5)P3 are plextrins and FOS. It also leads to releasing AKT from cell membranes. AKT is inactivated by PDK-1 in the phosphorylation process (17). AKT is responsible for passing to the G2/M phase of the cell cycle. It may also increase the expression of AKT in response to the cancer cells blocking the G2/M phase with paclitaxel. In cultures, the advantage of apoptotic over proliferation processes was observed. It indicates that activation of AKT is not persistent and causes only induction signals to resistance, but does not evoke active process. AKT cause inhibition of activity of phosphoinositolo-kinase (18). The mentioned kinase activates a great number of different factors, which is the reason for excessive proliferation and surviving (19). Additionally, AKT decreases the activity of P21 and P27 (20). This process can indicate that paclitaxel influences the proliferation processes in the G1 phase.

Another proliferation factor is the gene ERB2 coding kinase receptor. Heterodimerisation ERB2 with other proteins leads to phosphorylation traces of thyrosin and creates binding places for SRC proteins. ERB-2 leads to activation proteins such as RAF, RAS, RAB, MAPK, cJUN, and ribosomal kinase is responsible for cell growth and transformation. In studies on MCF-10A cell line, it was found that the insertion of the cERB-2 gene and mutated cH-RAS leads to the induction activity of MDR-1 and P glikoprotein. It causes increase of resistance for doxorubicin. Contrary to this excessive activity ERB-2, 3, 4 and EGF cause alteration in expression of different genes and was a reason for sensitivity to treatment. A gene responsible for cell transport is ABL. It also influences the regulation of factors connected with transport (21,22). The ABL gene product cooperates with microtubules and influences ENA - engaged in intracellular motion (23,24). ABL inhibits activation of ENA and lowers the dimerisation factor with KHC (25). It is the reason for the increase in activity of kinesin 1 and the acceleration of polymerization F actin. ABL, influenced by the microtubular transport protein, cooperates with their end C plus and coordinates the response to actin and microtubules on intracellular signals. It is possible that this cooperation is crucial in modeling the intracellular transport. Lowering transport may be the reason for the decrease in activity of different cytostatic drugs.

RAF is a transcriptor factor activating in a metabolic pathway RAF/MEK/ERK (26). This protein cooperates with different factors such as P21 and MYC. RAF/MEK/ERK pathway influences on P glikoprotein which inducts resistance for cytostatic treatment. Acceleration of transcription of Bcl-2 in relation to RAF/MEK/ERK results from phosphorylation of P-90 and activates CREB. In the next stage, CREB accelerates transcription of Bcl-2 by binding with its promotor (27). Mutations in cERB-2 gene lead to irregularity in cERB-2 protein what activates RAF. This can be the reason for resistance to treatment. Contrary to this process, RAF can inhibit the anti-apoptotic properties product of the BCL-2 gene through phosphorylation (28,29). Metabolic pathways increasing proliferation and inhibition of apoptosis activated by RAF are in advantage. A great number of pathways, activated by the production of the RAB gene, indicates an effectiveness of the proliferation process in the tested cells.

Another transcription factor, PIM1 joined with CD40, enables connections with TRAF 2-6 domens and is the reason for surviving and proliferation. It cooperates with NF-kB and different kinases such as ERK, cJUN, P38. In cell cultures of FDCP-1 line it causes inhibition of apoptosis through inactivation of cytokins, endonucleases and BAX. PIM1 cooperates with cMYC which induces the rise of leukemias (30). This protein cooperates with cMYC and cMYB, which is the reason for the acceleration of growth and the survival of damaged B lymphocytes. Some of the oncogenes accelerate proliferation which can influence the other genes and create a positive feedback loop which accelerate transformation and may be the reason for the decrease in controlling growth and division of cells in the apoptosis process. It seemed that such action exerted PIM1 on the cultures.

In the group of oncogenes, genes were identified to play a key role in the activation of apoptosis. An example of such gene is DAXX. The main function of this gene is to induce morphologic changes in cells. Production of this gene facilitates the release of cytochrom c, and activates many caspases (31). An additional function is connected with cytoplasm domen of FAS, which induces the rise of FADD domen (32). Product of Daxx gene cooperates with many transcription factors: PAX-5, ETS-1 and causes their repression (33). This protein induces deacetylation of histone proteins and inhibition of transcription processes (34). DAXX, joined with H2a, H2b, H3 and H4 histones, also with histone deacetylase - a basal factor, participates in repression of transcription (35). Non-phosphorylated form of 70 kDa Daxx participates in inhibition of transcription due to cooperation with histon deacetylase, DEK proteins and histone coplex (36). The direction of these activities is probably dependent on post-transcriptional modification. In some cases it can increase the production of the same gene.

Oncogenes belong to transcriptor factors that are AP-1 encoded by JUN family genes. The AP-1 Group consist of cFOS proteins creating JUN complexes. AP-1 cooperates

with transcriptor factors: ATF-2, NFAT. They participate in the proliferation and transformation processes. Activation of AP1 is strictly connected with the cooperation of P38. P38 which exists in some isoforms (37).

RAB proteins are small GTP-ases. They belong to oncogenes coding products interacting with proteins located on the inner surface of the cell membrane (38). Their expression is regulated by NF-E2. This factor also regulates activity of beta tubulin – a protein cooperating with paclitaxel. Paclitaxel can accelerate transcription of genes ranking the RAB family. Production of these genes inhibits activity of proteins connected with microtubules and the cytoskeletal system of the cell, formed by actin. They control the cell cycle and intracellular transport in cytoplasm. In the nucleus, they participate in forming nucleosomes and organize the mitotic spindle. They also play a crucial role in the activation of extracellular antigens (39).

Rab27A is associated with invasive and metastatic potential of human breast cancer cell development. The over expression of Rab27A protein redistributed the cell cycle and increased the invasive and metastatic abilities in breast cancer cells both in vitro and in vivo. We also certified that Rab27A bestowed the invasive and metastatic phenotypes on breast cancer cells by promoting the secretion of insulin-like growth factor-II (IGF-II), which regulates the expression of p16, vascular endothelial growth factor, matrix meta-metalloproteinase-9, cathepsin D, cyclin D1, and urokinase-type plasminogen activator. The data provides functional evidence that Rab27A acts as a novel mediator of invasion and metastasis promotion in human breast cancer cells, at least in part, through regulating the secretion of IGF-II, suggesting that synergistic suppression of Rab27A and IGF-II activities holds a promise for preventing breast cancer invasion and metastasis (40).

The signaling and in vitro phenotypic consequences of Rab11a expression and function were studied. Transfection of DN-Rab11a increased Erk1/2 activation downstream of EGF, but exerted no effect on the AKT pathway. Expression of DN-Rab11a inhibited MCF10A proliferation by 50-60%, and also inhibited anchorage-dependent colonization. Notably, DN-Rab11a transfection increased motility toward EGFR ligands. The data provides a first demonstration that Rab11a modulates EGFR recycling, and promotes the proliferation but inhibits the motility of an immortal breast line, consistent with the DCIS phenotype (41).

Once candidate genes are identified, we perform functional genomics by manipulating levels in normal and tumor cells using RNAi or transfection, and assessing a battery of cellular functions including proliferation, anti-apoptosis, loss of contact inhibition, changes in cell signaling or transcriptional profiles, anchorage-independent growth, and in vivo tumor growth. We have successfully used this approach to identify the RAB25 gene that has been implicated in the progression and aggressiveness of ovarian and breast cancers (42).

Important processes in which RAB participate are in many points convergent by interaction with paclitaxel. Cooperation of paclitaxel with RAB proteins seemed to be the opposite in the majority of points. Additionally paclitaxel in self-induction process activate excessive transcription and increase production activity of the RAB gene. It suggests development of effective resistance on applied drug in the positive feedback loop mechanism. Another aspect is external antigen induction by RAB proteins. It brings a conclusion that the RAB gene can be an indicator of cancer cell sensitivity on different schemes of cytostatic treatment. Additionally because RAB increases neoplasm antigen activity, it is possible to estimate changes in expression level of this gene which can be early manifestation of neoplasm development.

In conclusion it should be stated that paclitaxel influenced cancer cells in different ways. On one hand the main activity is concerned with activation of apoptosis and stopping cell division in G2/M cell cycle. On the other hand, increased resistance for this treatment is manifested by a significant increase of expression level of oncogenes. It seemed that in estimation response for cytostatic treatment it is dependent on answering a question which of these ways play a crucial role in development of persistent resistance.

RAB25 is likely to be an important factor in breast cancer development. RAB25 could be used as biological marker of breast cancer and provides a target for gene replacement therapy.

Changes correlated with a worsened outcome in both diseases. In addition, enforced expression of RAB25 in both breast and ovarian cancer cells decreased apoptosis and increased proliferation and aggressiveness in vivo, potentially explaining the worsened prognosis. A better understanding of genetic alterations as well as the physiologic and pathophysiologic roles of RAB GTPases may open new opportunities for therapeutic intervention and better outcomes.

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