Short communication

THE DETERMINATION OF CHANGES IN THE EXPRESSION OF GENES FOR SELECTED SPECIFIC TRANSCRIPTIONAL FACTORS IN in vitro DUCTAL BREAST CANCER CELLS UNDER THE INFLUENCE OF PACLITAXEL

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Abstract: This study aimed to determine the changes in the expression of genes for selected specific transcriptional factors that have both activating and repressing functions in in vitro ductal breast cancer cells, under the influence of paclitaxel, applying the microarray technique. The cells are treated with 60 ng/ml and 300 ng/ml doses of paclitaxel that correspond to those applied in breast...
cancer therapy. About 60 ng/ml doses of paclitaxel cause a statistically significant increase in expression of all the 16 analysed genes coding transcriptional factors, ranging from 1.84-fold (for PO4F2) to 4.65-fold (for LMO4) (p < 0.05) in comparison with the control cells, and enhanced the taxane mechanism of action. The 300 ng/ml doses of paclitaxel cause a cytotoxic effect in the cells. In this article, we argue that these changes in gene expression values may constitute prognostic and predictive factors in ductal breast cancer therapy.

**Key words:** Breast cancer, Transcriptional factors, Gene expression, Microarrays, Anti-tumour, Treatment, Paclitaxel, Microtubules, Cytotoxic effect, Apoptosis

**INTRODUCTION**

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths among women worldwide. However, due to the multi-factor and multi-stage nature of mammary tumourigenesis, the successful diagnosis and treatment of breast cancer remains a difficult task for oncologists [1]. Death caused by this disease is generally associated with metastasis to other organs, making it similar to other cancers. Breast cancer is also the main driving factor for the large number of deaths recorded among women in Poland. The incidence of breast cancer increased by about 5% in Poland in the last few years. This cancer comprises 20% of all cases of malignant tumours. In recent years, more and more women aged 35-39 years old suffer from this epidemic [2].

Cancer of the breast and other epithelial organs typically exhibits both a longer period of development and a greater phenotypic diversity. The well-described biological and clinical heterogeneity of breast tumours is thought to result from acquired or inherited gene defects [3]. Ductal carcinoma **in situ** (DCIS) of the breast represents a group of non-invasive breast tumours commonly detected in women undergoing screening mammography. DCIS is characterised by malignant epithelial cells accumulating in the ducts of the breast without invading through the basement membrane into the surrounding tissue [4]. Patients diagnosed with ductal carcinoma in situ (DCIS) at a young age appear to have different natural history and biology, including a higher local relapse rate, compared to patients diagnosed later in life [5].

Transcriptional factors tightly control oncogene expression. Accurate gene expression regulation is the most fundamental process in biology which influences the cells of organisms. Mistakes in gene activation and the development-specific and tissue-restricted gene programmes under the control of transcriptional factors represent a rich and diverse source of mechanisms which, if disrupted, can lead to occurrence of malignancy, including breast cancer. A known example is oncogene expression, where the effect of one gene transcription may be tumour cell transformation. Many gene stimulating or repressing factors have been shown to have oncogenic properties when genomically altered (mutated, rearranged, amplified or deleted), transcriptionally
upregulated, or post-translationally modified. Many of the well-studied proto-oncogenes and tumour suppressor genes (anti-oncogenes) are known to encode proteins which, alone or in a complex with other factors, act as transcriptional regulators; these include some of the earliest identified oncogenes (Myc and Myb) and tumour suppressor proteins (Rb, p53), and the Wilms’ tumour gene product WT1. Nowadays, one of the most common medicines for breast cancer therapy is the anti-mitotic paclitaxel (PTX) – an alkaloid, diterpene, lipophilic, not soluble in water. Paclitaxel’s antitumour mechanism of action is connected with making complexes with tubulin monomer, which is the main mitotic spindle protein, and with stabilization of non-functional microtubule wisps. The disturbance of balance between tubulin dimers and microtubules towards the latter causes inhibition of the cell cycle at the G2/M checkpoint and proliferation. The cytotoxic effect is associated with paclitaxel positive regulation of proapoptotic proteins Bak and Bax, with negative regulation and inactivation of antiapoptotic protein Bcl-2. According to earlier studies, signal processes strengthened by paclitaxel may induce apoptosis in a number of mechanisms: mitogen-activated protein kinase pathway, A kinase pathway, Raf-1/ras/Bcl-2 pathway. In contrast to other antitumour treatments, paclitaxel has no genotoxic activity. Besides anthracyclines, the taxanes are among the most effective agents in mammary gland cancer chemotherapy and cause 30-60% remission in monotherapy and even 90% in polytherapy [6-12].

The microarray technique opens new perspectives in medicine and pharmacy. The characterisation of the gene expression profile in the pathological state makes it possible to diagnose and predict the course of the disease quicker and more effectively as well as to recommend a good medicine for the individual patient’s needs independently. Microarray technology can provide many types of information to help drug discovery and development. In their simplest application, microarrays can be used to screen for changes in gene expression following exposure of tumour cells to drugs either in culture or in patients following treatment. Studies of this type may provide information on the precise mechanism of action of the drug or could lead to the identification of early markers of drug response. For predicting the longer-term clinical response to drug treatment, microarray information could also yield information about possible side effects, and identify markers that, in a clinical context, could be used to predict possible adverse events. This technology can also provide information for identifying and validating new therapeutic targets. Microarray expression patterns could be integrated on the one hand with clinical data to identify new markers to predict biological behaviour of tumours, and on the other hand with databases of drug sensitivity to unravel the molecular basis of drug action [13].

The central goal of this study was to determine the changes in the expression of genes for selected specific transcriptional factors that have both activating and repressing functions in \textit{in vitro} breast cancer cells under the influence of paclitaxel.
MATERIAL AND METHODS

The cells under study were taken from mammary gland tissues from 36 patients of Lublin Oncology Centre, Poland, with diagnosed ductal breast carcinoma during mastectomy. The patients were not cured earlier. The material was histopathologically verified and included cases of breast cancer in stage I and II according to the Bloom classification (pre-invasive cancer, tumours up to 2 cm, no lymph node metastasis). The material (tissue) examined was homogenized mechanically and next enzymatically (0.01% trypsin, Sigma). Then the cells of ductal breast carcinoma were seeded in a Falcon bottle of 25 mm² wells in RPMI (Sigma) medium with addition of 5% fetal bovine serum (FBS, Sigma), penicillin and streptomycin, and cultured at 37°C, in an atmosphere of 5% CO₂ and 90% humidity. The cells that reached the density of 10 000 cells/ml were treated with paclitaxel (TAXOL®, Bristol-Myers Squibb; England) in 60 and 300 ng/ml doses and incubated for 72 h. The added paclitaxel 60 ng concentration was calculated on the basis of a therapeutic dose that was 175 mg/m² of body surface area in relation to the surface of the used Falcon bottle. The value of the second, fivefold higher dose of 300 ng was the result of dose assessment for six chemotherapy cycles applied on average (3-9) in breast cancer treatment. After 72 h of incubation, the cells formed a monolayer. The units were expressed as weight/volume units: 60 ng and 300 ng per millilitre of culture medium. Simultaneously, the control epidermal cell cultures of ductal breast cancer were incubated without paclitaxel but with 5% dimethyl sulfoxide (DMSO).

The total RNA from control ductal breast cancer cells, including those after treatment with paclitaxel, was isolated by using a TRI (Sigma) kit according to the manufacturer’s instructions and kept until the next steps of the study at -70°C. Complementary DNA (cDNA) with radioactive labelled ³²P dNTPs obtained in a reverse transcription reaction by using a standard kit (Sigma) was cleaned on Sephadex G-25 and hybridized to the microarray (Panorama Human Cancer cDNA Labeling and Hybridization Kit; CDLBL-HCN, Sigma-Genosis). The samples with a mixture for reverse transcription reaction RNA from a model gene of Escherichia coli (from Panorama Armored RNA E. coli-B 1444 RNA kit) were added in order to make possible the calibration of expression of examined genes in relation to the expression of this model gene on the microarray. The hybridization buffer contains the test DNA of salmon (Sigma-Genosis Kit CDBL-HCN). The hybridization was performed in the hybridization oven Bachover GmbH-D7410. Next the gamma radiation from points on the microarray referring to the examined genes was captured (radiation detector Screen Imaging K, Bio-Rad) for 24 hours. The gamma radiation caused chemical changes of the carrier (fluorobromide salt base with Europium on the surface) sensitive to the radiation sited in the detector box. Then the carrier was scanned on Molecular Image FX (Bio-Rad) with resolution of 50 microns. As a result, the picture of expression of the examined genes on the microarray was received and transmitted as a file on a computer disk. The expression from the
individual points of microarrays normalizes with the expression level of the model \textit{E. coli} gene localized at the control points. The gene expression results were analysed using the computer program ‘\textit{Quantity One – version 4.2.1}’. The value of the individual gene expression is assessed as the average pixel count for the determined unit of space corresponding to the localization of a single gene on the microarray. The gene expression results from the cells treated with examined doses of paclitaxel were compared with the level of expression of analysed genes from the control cells. The studies were performed in triplicate using one set of reagents.

**Statistical analysis**

The expression results of 16 genes for transcriptional factors that have the activity of both transcriptional activator and repressor were statistically analysed with Statistica 6.0.

1. The value of medium level of expression and standard deviation for each examined gene were assessed.
2. The data were compared statistically using Student’s t-test. A probability value of $p < 0.05$ (marked as *) indicates a statistically significant difference.

Tab. 1. List of studied genes for transcriptional factors that have both activating and repressing activities.

| Gene   | Full name                                                                 |
|--------|---------------------------------------------------------------------------|
| 1 TFAP2A | transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) |
| 2 CTCF   | CCCTC-binding factor (zinc finger protein)                                 |
| 3 MBD2   | methyl-CpG binding domain protein                                         |
| 4 RUNX   | runt-related transcription factor 2                                       |
| 5 LMO4   | LIM domain only 4                                                         |
| 6 YY1    | YY1 transcription factor                                                  |
| 7 TFAP2C | transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma) |
| 8 PDEF   | SAM pointed domain containing ets transcription factor                    |
| 9 KLF4   | Kruppel-like factor 4 (gut) Kruppel                                       |
| 10 MADM4 | SMAD4 SMAD, mothers against DPP homolog 4 (Drosophila)                    |
| 11 PHB    | prohibit                                                                   |
| 12 TP63  | P63 tumour protein p63                                                    |
| 13 TP73  | TP73L tumour protein p73-like                                             |
| 14 POU4F2| POU domain, class 4, transcription factor 2                                |
| 15 HOXA5 | homeo box A5                                                              |
| 16 HOXA1 | homeo box A1                                                              |
RESULTS

In this study, we applied the microarray technique to determine the expression of 16 genes for specific transcriptional factors in \textit{in vitro} breast cancer cells under the influence of paclitaxel. Among all of the genes studied on the microarray used (Tab. 1), only these 16 selected genes code transcriptional factors that have activating and repressing functions in breast cancer cells. All genes have a very high statistically significant level of expression in the \textit{in vitro} ductal breast cancer cells treated with 60 ng/ml doses of paclitaxel in comparison to the control cells.

The second, 300 ng/ml doses examined cause a cytotoxic effect in the \textit{in vitro} ductal breast cancer cells. A statistically significant decrease in expression of 5 genes was observed when compared with the control cells untreated with paclitaxel (Fig. 1).

Fig. 1. Arithmetic means and standard deviations of expression values for all 16 studied genes (Tab.1.) coding for both activating and repressing transcriptional factors in ductal breast cancer control cells \textit{in vitro} and treated with 60 ng/ml and 300 ng/ml doses of paclitaxel.

Fig. 2. Statistically significant values (*\(p < 0.05\)) of increased level of expression of 16 genes (Tab.1) in ductal breast cancer cells \textit{in vitro} treated with 60 ng/ml of paclitaxel in comparison to control cells.
The expression increase for the genes in the breast cancer cells under the influence of the 60 ng/ml dose of paclitaxel studied was in the range of 1.84–4.65-fold, in comparison with the control cells. The highest expression increase (4.65-fold) was noted for the LMO4 gene and the lowest (1.84-fold) for PO4F2 (Fig. 2).

DISCUSSION

The essence of any organism is the spatial and temporal expression pattern of its gene repertoire. While the genome provides the template, it is the way genes are expressed that defines the organism. Consequently, regulation of gene expression influences almost all biological processes in an organism. Transcription factors (TFs) are often termed the master regulators of gene expression. By binding to the DNA, they tightly control where and when the nearby target gene is expressed. Mistakes in gene activation caused by too high or too low expression of a particular gene may lead to occurrence of the disease. The best known example is oncogene expression, where the result of one gene transcription may be tumour transformation [14, 15]. The increasingly popular microarray technique opens new perspectives in medicine and pharmacy. The characterisation of gene expression profile in pathological states makes it possible to diagnose and predict the course of disease quicker and more effectively, as well as to choose a good medicine independently of the individual patient’s needs [16, 17]. There is a need to identify novel breast tumour-associated molecules as diagnostic/prognostic markers of breast cancer as well as to discover targets of vaccines and drugs against this cancer.

It has been shown that paclitaxel-treated cells have defects in mitotic spindle assembly, chromosome segregation, and cell division. Paclitaxel stabilizes the microtubule polymer and protects it from disassembly as well as cooperating with microtubule associated proteins (MAPs). This influences cell cycle proteins and the apoptotic pathway [8, 18]. As a result, one could expect that the presence of paclitaxel in the breast cancer cells under study will have a significant role in their cell cycle and metabolism.

We observed the highest (4.65-fold) increase in LMO4 expression in ductal breast cancer cells treated with the 60 ng/ml doses of paclitaxel in comparison with the control cells. The LMO4 gene, widely expressed in both embryonic and adult tissues, codes a transcriptional cofactor, LMO4, that primarily functions as a docking site for other factors and contributes an activation or repression domain to influence transcriptional activity. LMO4 functions as a protein–protein interaction surface. According to Visvader J.E. et al. (2001), overexpression of the LMO4 gene is found in more than 50% of breast cancer cell lines and primary breast cancers, suggesting that its protein product contributes to the pathogenesis of breast cancer. Increased LMO4 expression is observed in pre-malignant tissue relative to normal breast. The study indicates that this gene may be an early marker in the progression of breast cancer because almost 40% of ductal carcinomas in situ expressed abundant LMO4 RNA. Although LMO4 is
often expressed in proliferating cells and organs, it is present in post-mitotic cells, which apparently is not a simple marker of cell growth. The molecular mechanism by which LMO4 participates in mammary gland development has not been elucidated [19]. LMO4 was initially identified in an expression screen by using serum from a breast cancer patient. Moreover, in breast cancer, LMO4 expression appears to be inversely correlated with oestrogen receptor expression. It has been shown that LMO4 forms a complex of co-repressor CtIP and BRCA1 in breast epithelial cells. Further study showed that LMO4 can repress BRCA1-mediated transcriptional activation [20]. Over-expression of LMO4 in non-invasive, immortalized human MCF10A cells promotes cell motility and invasion. Studies conducted by other authors show that a high nuclear level of LMO4 is an independent predictor of death from breast cancer in a cohort of 159 patients with primary breast cancers. Together, these findings suggest that deregulation of LMO4 in breast epithelium contributes directly to breast neoplasia by altering the rate of cellular proliferation and promoting cell invasion [19]. Taking into consideration other authors’ and our results, it is very interesting to observe the very high increase in LMO4 gene expression level in \textit{in vitro} ductal breast cancer cells treated with a 60 ng/ml dose of anti-mitotic paclitaxel. Thus it can be stated that the final apoptotic effect in these cells is the result of complex co-operation between pro-apoptotic and anti-apoptotic genes and proteins. Further studies should be performed to explain these mechanisms in detail. In our study, the expression of AP2A was 3.86-fold and AP2C 2.02-fold higher in the breast cancer cells treated with 60 ng/ml doses of paclitaxel than in the control cells. Expression microarray results in human breast carcinoma cells indicate that genes for activating enhancer-binding protein 2alpha (AP-2alpha) and activating enhancer-binding protein 2gamma (AP-2gamma) are targets for transcriptional activation by p53, and suggest that AP-2 proteins may mediate some of the downstream effects of p53 expression, such as inhibition of proliferation [21, 22]. Expression of either AP-2alpha or AP-2gamma inhibits growth of human breast carcinoma cells \textit{in vitro}. It is known that AP-2gamma significantly up-regulates p21 mRNA and proteins, inhibits cell growth, and decreases clonogenic survival. Cell cycle analysis reveals that forced AP-2gamma expression induces G1-phase arrest, and decreases DNA synthesis including the fraction of cells in the S phase. AP-2gamma expression leads to cyclin D1 repression, decreases Rb phosphorylation, and decreases E2F activity in breast carcinoma cells [23]. A relatively high increase in the expression of CTCF (2.99-fold) was observed in breast cancer cells incubated with 60 ng/ml of paclitaxel. Rakha \textit{et al.} (2004) detected a correlation between expression of CTCF and histological grade where a high percentage of low-grade tumours that have a less proliferative activity show positive nuclear expression, while the high-grade tumours mainly show reduced or absent expression. They found a correlation between positive cytoplasmic expression and tumours of smaller size. These results are consistent with the previous reports that showed the ability of CTCF to inhibit cell growth
and proliferation. No significant correlation between CTCF expression and other prognostic markers of breast cancer such as lymph node stage or with patient outcome was found [24]. It was observed that a loss of BRCA1 correlates with the inappropriate cytoplasmic expression of CTCF in tumours that lack or express low levels of BRCA1. As a consequence, loss of BRCA1 expression may lead to the deregulation of numerous cell functions and chromosome instability, which together predispose to the formation and progression of sporadic breast tumours [25].

As a transcription factor, Brn-3b mediates its effect in mammary epithelial cells by regulating gene expression, either directly – repressing the activity of the BRCA1 promoter – or indirectly. A strong correlation was found between elevated levels of the Brn-3b POU transcription factor and high levels of HSP-27 protein in manipulated MCF-7 breast cancer cells as well as in human breast biopsies, and conversely, on loss of Brn-3b the level of HSP-27 was decreased. Brn-3b via interaction with the oestrogen receptor can directly and indirectly activate HSP-27 expression. This may represent one mechanism by which Brn-3b mediates its effects in breast cancer cells [26].

Expression of PDEF in breast cancer cells leads to inhibition of invasion, migration, and growth. PDEF protein is reduced in human invasive breast cancer, and is absent in invasive breast cancer cell lines. Expression of PDEF therefore results in the down-regulation of urokinase-type plasminogen activator and activation of the promoter of the tumour suppressor gene, Maspin. Growth-suppressive effects of PDEF expression are mediated in part by a G0-G1 cell cycle arrest associated with elevated p21 levels. These results from other studies indicate that PDEF loss may alter the expression of genes controlling progression to invasive breast cancer [27, 28]. Collectively, the over-expression of PDEF observed in breast cancer cells treated with 60 ng/ml doses of paclitaxel in our study seems to agree with its anti-mitotic and anti-angiogenic mechanism of action.

In the examined cells stimulated with paclitaxel, there is a 2.86-fold increase of RUNX2 gene expression relative to the control cells. The breast cancer cells preferentially metastasise to the bones. RUNX transcription factors comprise a family of proteins that are essential for organogenesis and a unique nuclear matrix-targeting signal in the C terminus which directs these factors to their appropriate subnuclear domains. At these sites, they interact with co-regulatory proteins and target genes. It has been previously shown that aberrant expression of the RUNX2 DNA binding domain in metastatic breast cancer cells can prevent production of osteolytic lesions in bone. It is obvious that proper RUNX2 subnuclear targeting is required for osteolysis. These findings suggest that fidelity of RUNX2 intranuclear organization is necessary for expression of target genes that mediate the osteolytic activity of metastatic breast cancer cells [29]. As the result of treating the ductal breast cancer cells with 60 ng/ml of paclitaxel there is an increase (1.96-fold) of KLF gene level. KLF4 (GKLF/EZF) encodes a transcription factor that has context-dependent oncogenic or tumour-
suppressor functions. KLF4-induced arrest is bypassed by the RAS target cyclin-D1, so inactivation of the cyclin-D1 target and the cell-cycle inhibitor p21CIP1 not only neutralizes the cytostatic action of KLF4, but collaborates with KLF4 in oncogenic transformation. On the other hand, KLF4 suppresses the expression of P53 by directly acting on its promoter [30].

Over half of human tumours, including breast cancer, are associated with mutations that directly affect retinoblastoma protein (pRb) and P53, or influence their pathways. PHB has been shown to co-localise with pRb, P53 and E2F in various cell lines. It is known that PHB is generally over-expressed in transformed cells compared with their non-transformed counterparts. These observations, together with the earlier ones that PHB functions as a tumour-suppressor protein and microinjection of PHB mRNA blocks cell proliferation, have led some investigators to suggestions that G1–S cell growth arrest is brought about by a process involving the repression of E2F-mediated transcription [31].

P63, as a member of the P53 gene family, is involved in cellular differentiation, expressed in the nuclei of myoepithelial cells of normal breast ducts and lobules. P63 and other myoepithelial cell markers have been described in matrix-producing and metaplastic carcinomas of the breast, suggesting that these tumours share myoepithelial cell differentiation. In mammary epithelium, P63 has been shown to be expressed only in the myoepithelial layer [32-34].

TP73, a structural and functional homologue of transcription factor P53, has the potential to activate TP53 target genes and to interact with P53, as well as inhibit cell growth in a P53-like manner by inducing apoptosis when overproduced. Under-expression or loss may contribute to arrest failure at cell-cycle check points [35].

Homeotic genes encode master regulatory transactivating proteins which are likely to regulate other genes within the mammary epithelium [36]. HOXA1 stimulates the transcriptional activation of a number of pro-oncogenic molecules, including cyclin D1 and Bcl-2, thereby permitting increased proliferation and survival of human mammary carcinoma cells [37], whereas deregulation of HOXA5 in breast epithelium can contribute to breast cancer biogenesis primarily by regulating the expression of the tumour-suppressor protein P53 and the progesterone receptor [38-40].

The MBD2 gene, encoding the methyl-CpG-binding domain, is a major methylation related gene and functions as a transcriptional repressor that can specifically bind to the methylated regions of other genes. MBD2 may also mediate gene activation because of its potential DNA demethylase activity. As the mechanism by which MBD2 acts to prevent transcription of GSTPI, and other genes inactivated by CpG hypermethylation in cancers, becomes more fully elucidated, perhaps new cancer treatment or prevention strategies might target the MBD2 transcriptional repression pathway. There are suggestions that GSTPI is silent in MCF-7 breast cancer cells as a result of CpG island hypermethylation. The repression of hypermethylated GSTPI CpG island alleles...
is at least partly mediated by MBD2 \[41, 42\]. It has been shown that YY1 and TGF\(\alpha\)/SMAD4 transcription factor activities may be significant in the progression of breast cancer \[43-45\]. Taking into account the above information, it may be said that the differences in the level of gene expression in breast cancer cells under study are the result of changes in activation of transcriptional factors caused by treating them with the 60 ng/ml doses of paclitaxel. Examined cells are sensitive to paclitaxel, and under the influence of this treatment, they start the processes leading to apoptosis. Thus, it is possible that the effect of treating the breast cancer cells with paclitaxel is the cooperation of genes from all groups of transcriptional factor repressors and activators and those functioning as repressors and activators. The 300 ng/ml dose turned out to be too concentrated and toxic for \textit{in vitro} ductal breast cancer cells, despite the fact that it was chosen on the basis of the treatment scheme for paclitaxel. As a consequence, a decrease in the expression of all studied genes when compared with the control cells and a cytotoxic effect were noted.

We can therefore presume that the changes in gene expression values may constitute prognostic and predictive factors in ductal breast cancer therapy. This study indicates that the analysis of transcriptional changes at the mRNA level may be indirectly used for the assessment of protein activity because the protein molecules are modified after the translation, which influences their cell localization, secretion and complex formation capabilities. The introduction of assessing the level of gene expression using DNA microarrays gives hope for finding a new prognostic factor for patients with breast cancer, including the separation of the groups of patients for whom a proper model of adjuvant therapy will be possible to work out \[46, 47\].

Identification of molecular alterations in the DNA copy number and expression profiles are useful to identify new chromosomal regions involved. The same applies to genes relative to the advancement of neoplastic processes as therapeutic targets \[48, 49\]. As it could be seen from this study and studies conducted by other researchers, the association between the genes are very complex, which is why getting to know how this machinery functions is one of the greatest challenges for biologists and medical scientists.

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