人類神經膠瘤血管生成相關因子及受體之表現與臨床病理的關係

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中文摘要

根據我們之前的研究顯示：血管生成因子 VEGF 的表現情形與神經膠瘤細胞株及檢體的惡性程度有相關性，然而，整體的基因群變化與神經膠瘤惡化程度之間的關係尚未十分明確。為了更進一步的研究基因群變化與腫瘤病理分類的關係，我們利用定量聚合鍵連鎖反應 (real time PCR) 及基因微陣列分析 (cDNA microarray) 方法，針對不同惡性程度的腫瘤組織研究其基因群變化。我們收集臨床接受開顱手術病患的腦瘤標本，包含低惡性度神經膠瘤 (第一度及第二度星細胞瘤)，高惡性度神經膠瘤 (第三及第四度)，以及腦膜瘤等三種不同種類的腫瘤檢體，並與癲癇手術切除之正常腦組織做比較。研究結果顯示：Nidogen 2、Calreticulin、CRMP3、Chaperonin、TIMP1 及 CD44 等基因，會隨惡性化程度提高表現量；MCF-2 cell line derived transforming sequence-like，Laminin alpha 4 及 CRMP2 等基因，則在惡性高的組織中表現量較低；此外，Chaperonin，Eukaryotic translation initiation factor 2B，CRMP3 及 CRMP1 等基因在高惡性度神經膠瘤的表現量多於低惡性度神經膠瘤及正常的組織；而 IL2、Homo sapiens BAC clone 則相反，表現量低於正常的組織。除此之外，我們也對神經膠瘤細胞株做研究，與腫瘤組織檢體相較之下，有些基因很少表現在細胞株，例如：Serum deprivation response (SDPR) mRNA、Thy-1 cell surface antigen、CRMP5、CRMP2、Adenomatosis polyposis coli down-regulated 1 (APCDD1) 及 LIM domain binding 2 等。我們期望，這些特殊的基因表現圖譜，可以幫助我們在臨床上判別腫瘤惡性的程度，或作為臨床治療的參考依據。

關鍵詞 (keywords)：血管生成、神經膠瘤、基因微陣列分析、腦膜瘤
Abstract

In our previous study, we found that the expression of VEGF by human GBM cell lines and specimens was positively correlated with the malignancy of gliomas. However, the gene expression profile of malignant brain tumors was not very clear. In this study, we investigated the relationship between gene expression profiles and clinical pathology of brain tumors. We used a home-made angiogenesis cDNA microarray and real time PCR assay to analyze and quantify the differential gene expression profiles in gliomas with different grade of malignancy. Three groups of brain tumor tissues, including low grade gliomas (grade I and II astrocytomas), high grade gliomas (grade III and IV), and meningiomas, were analyzed and the results were compared with those of normal brain tissues from patients received an epilepsy surgery. We found that Nidogen 2, Calreticulin, CRMP3, Chaperonin, TIMP1 and CD44 had a tendency of increasing while MCF.2 cell line derived transforming sequence-like, Laminin alpha 4, and CRMP2…..etc had a tendency of decreasing as the malignancy of tumor increased. Besides, we also found that the expressions of Chaperonin, Eukaryotic translation initiation factor 2B, CRMP3, CRMP1 were higher in high grade gliomas than those of low grade gliomas and normal tissues. The expressions of IL2, Homo sapiens BAC clone were lower in high grade gliomas. We also investigated the gene expression profiles of brain tumor cell lines. Several genes were poorly expressed in cell lines such as Serum deprivation response (SDPR) mRNA, Thy-1 cell surface antigen, CRMP5, CRMP2, Adenomatosis polyposis coli down-regulated 1 (APCDD1), and LIM domain binding 2. We demonstrated that the differential gene expression profiles could be used to distinguish the grades of gliomas in this study, and hope that this might become an importance reference in clinical and pathological diagnosis of brain tumors and in deciding the appropriate strategy of treatments in brain tumors.

關鍵詞(keywords): angiogenesis, glioma, cDNA microarray, meningioma
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前言(Introduction)

GBM, which is synonymous with grade IV astrocytoma, is considered to be one of the most malignant human neoplasms [1]. Pilocytic (grade I) astrocytomas have a more favorable prognosis than diffuse astrocytomas and are characterized by a more circumscribed growth pattern with a limited infiltrative behavior and infrequent anaplastic progression [2]. Primary malignant brain tumors tend to regrow regardless of the treatment administered. Additionally, differentiated lower-grade gliomas may during the course of time undergo anaplastic development and become dedifferentiated fast-growing neoplasms. At the present time the fundamental mechanism that triggers the cascade of events that lead to the progression of an initially low-grade quiescent or slow-growing glioma to a highly proliferative anaplastic tumor with a dense three-dimensional capillary network is not known [3]. Although differentiated low-grade gliomas may reach an impressive volume without showing morphological signs of anaplasia, it seems that a high proliferative rate of tumor cells requiring an increased blood supply is not possible without concomitant acceleration of angiogenesis [4]. Proliferation and angiogenesis are important prognostic variables in high grade gliomas. To produce a blood vessel, endothelial cells must divide, migrate, degrade the extracellular matrix, and differentiate to a tube-like structure. This multistep process is controlled by a large number of distinct positive and negative regulatory molecules [4]. These molecules may be associated with tumorigenicity and angiogenesis such as vascular endothelial growth factor (VEGF), acid and basic fibroblast growth factor (aFGF & bFGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α & TGF-β), tumor necrosis factor alpha (TNF-α), angiopoietin-1, angiopoietin-2 (Ang-1 & Ang-2) etc. They may play a role when produced by the tumor cells themselves (autocrine loops) or by stromal cells (paracrine loops).

The extensive heterogeneity of tumors has made their pathological classification rather difficult. To date, few studies have been reported about brain tumor classification by gene expression patterns[5, 6]. A study uncovered six genes, including TIMP3, EGFR, and GDNPF, that were expressed in 64–100% of grade II tumors and were not expressed in three non-tumor tissue samples derived from three different brain regions. It was also reported that seven genes, including PDGFR-α, PTN, LRP, and SPARC, were up-regulated by at least 2-fold in 20–60% of the grade II tumors compared with the non-tumor brain tissue samples[7-10]. Although several genes have been identified to be associated with tumorigenesis and anaplastic progression of gliomas, their contribution to the molecular classification of tumors has been limited. Molecular expression profiles using cDNA-based microarrays have been used to derive a molecular-based classification of several cancer types including B-cell lymphomas, malignant melanomas, and breast cancer[11-13]. DNA
microarrays has been used mainly to study gene expression in response to physiological conditions and alterations of the expression pattern in disease states.

研究目的(Purpose of Study)

In this study, we used an angiogenesis microarray and found it useful in profiling the gene expression of brain tumors, which may become a new method for studying molecular pathology of tumors. The aim of this study is to investigate and correlate the expression of a broad panel of angiogenesis related factors and receptors in gliomas to the degrees of malignancy. The relationships between the amount of various growth factors expressed and degrees of malignance were tested. The understanding of the role of growth factors and angiogenetic factors in gliomas would not only increase our knowledge of the pathogenesis of tumor but might also offer novel opportunities for therapeutic intervention.

文献探討(Review of literatures)

Primary malignant brain tumors tend to regrow regardless of the treatment administered. Additionally, differentiated lower-grade gliomas may during the course of time undergo anaplastic development and become dedifferentiated fast-growing neoplasms which have the following characteristics: high cellularity, nuclear polymorphism, increased mitosis, tissue necrosis, sprout of small vessels, and proliferation of vascular endothelial cells [14, 15]. At the present time the fundamental mechanism that triggers the cascade of events that lead to the progression of an initially low-grade quiescent or slow-growing glioma to a highly proliferative anaplastic tumor with a dense three-dimensional capillary network is not known. Although differentiated low-grade gliomas may reach an impressive volume without showing morphological signs of anaplasia, it seems that a high proliferative rate of tumor cells requiring an increased blood supply is not possible without concomitant acceleration of angiogenesis [16]. Proliferation and angiogenesis are important prognostic variables in high grade gliomas. To produce a blood vessel, endothelial cells must divide, migrate, degrade the extracellular matrix, and differentiate to a tube like structure. This multi-step process is controlled by a large number of distinct positive and negative regulatory molecules [4]. These molecules may be associated with tumorigenecity and angiogenesis such as vascular endothelial growth factor (VEGF), acid and basic fibroblast growth factor (aFGF & bFGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor alpha and beta (TGF-α & TGF-β), tumor necrosis factor alpha (TNF-α), angiopoietin-1, angiopoietin-2 (Ang-1 & Ang-2) etc [17]. They may play a role when produced by the tumor cells themselves (autocrine loops) or by stromal cells (paracrine loops).

Vascular endothelial growth factor (VEGF) is a potent and specific endothelial cell mitogen in vitro, induces angiogenesis in vivo [18] and produces a profound increase in
vascular permeability[19]. It is identical to vascular permeability factor [20, 21]. The biological effects of VEGF are mediated mainly by two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/flk-1), which are expressed almost exclusively in endothelial cells [22]. Two biologic characteristics of VEGF are unique among angiogenesis factors. VEGF is a direct and specific endothelial cell mitogen. Other angiogenesis factors either support angiogenesis indirectly (epidermal growth factor/EGF, tumor necrosis factor α, transforming growth factor β1, or angiogenin) or are active on non-endothelial cell types (acid fibroblast growth factor/aFGF, or basic fibroblast growth factor/bFGF) [23]. VEGF is also a secreted protein in contrast to aFGF, bFGF and platelet-derived endothelial cell growth factor which lack signal peptides required for extracellular transport. Supposedly, these latter factors are released by leakage from damaged cells or bound to secreted extracellular matrix molecules. We have shown that VEGF is detected in glioma cell cultures by ELISA[24] and hypothesize that VEGF is the central mediator of glioma angiogenesis because of its characteristics[25] and its ability to initiate the sequence leading to neovascularization [19].

Three of the major histopathologic features of malignant glioma (hypervascularity, tumor necrosis, and peritumoral brain edema) may be related to VEGF. Glioblastoma multiforme thus seems to be the prototype tumor suitable for anti-angiogenic therapy [26]. Definitive studies of regulatory pathways controlling glioma-derived VEGF production may lead to development of new therapeutic strategies for this common malignant brain tumor. We have demonstrated that three tyrosine kinase receptor growth factors, EGF, bFGF and PDGF-BB, are each capable of inducing VEGF production from glioma cells [24]. The combined effects of these growth factors were additive at low concentrations but reached a maximum stimulatory effect at high concentrations implying a convergent pathway leading to enhanced VEGF secretion.

Recently, three major techniques are developed to monitor thousands of gene expression. The first is a cDNA microarray developed by Brown et al., which is based on two probes (labeled with different fluorochrome) hybridization on glass microscope slide to which PCR-amplified fragments of cDNA are dotted [27]. After laser scanning, emitted fluorescence are collected to quantify gene expression. The secondary is the serial analysis of gene expression (SAGE) developed by Kinzler et al., which uses specific restriction endonuclease (FokI and NlaIII) and linkers to form serial link tags, amplified by PCR with linker-specific primers, and then sequenced [28]. By means of the possibility of appearance for 9-bp tags, it is able to estimate gene expression. The third is the DNA chip method derived from oligonucleotide array. The oligonucleotide containing specific sequence are synthesized on glass directly and hybridized with fluorescence-labeled probe, analyzed by the signal of the hybridized oligonucleotides.
Total RNA was isolated from surgical specimens of glioma and meningioma tissues which were collected intraoperatively and were immediately snap-frozen in liquid nitrogen before being stored at -80 °C. RNA (5 µg) was reverse transcribed, using random hexamer primers (50 pmol; Perkin Elmer Cetus, Norwalk, CT) and 200U Moloney murine leukemia virus (MoMuLV) reverse transcriptase (Perkin Elmer Cetus), at room temperature for 10 min in 1X buffer (10 mM Tris-HCl, 50 mM KCl), 0.5 mM each dATP, dCTP, dGTP, 40 µM dTTP, 40 µM biotin-16-dUTP, 10 mM DTT, and 0.5 unit/µl RNAse inhibitor. The reaction was then carried out at 42 °C for 90 min, and followed by a termination step at 99 °C for 5 min. Following this, 5.5 µl 3 M NaOH was added to lysis ribonucleotides at 50 °C for 30 min, and then neutralized with 5.5 µl 3M CH3COOH. Subsequently, 35 µl deionized water, 50 µl 7.5 M ammonium acetate, 5 µl carrier (linear polyacrylamide, 4 µg/µl), and 375 µl 100% alcohol were added. The reaction mixture was mixed and put undisturbed at -80 °C for 30 min, and centrifuged (14,000 rpm) for 20 min in order to precipitate the single strand cDNA probe. It was then washed with 70% alcohol and dried, dissolved in 5 µl or appropriate amount of dH2O.

The nylon membrane carrying the double-stranded cDNA targets was pre-hybridized in 1 ml (option) hybridization buffer (5× SSC, 0.1 % N-lauroylsarcosine, 0.1% SDS, 1% BM blocking reagent made by Boehringer Mannheim, and 50 µg/ml salmon sperm DNA) at 68°C for 1 h before hybridization was carried. The cDNA probes were mixed in 10 µl hybridization buffer (5x SSC, 0.1 % N-lauroylsarcosine, 0.02% SDS, 200 µg/ml d(A)10, 400 µg/ml human COT1 DNA, and 1% BM blocking reagent) to prevent non-specific binding and were hybridized to the cDNA fragments on the membrane by Southern hybridization procedure. The 10-µl reaction mixture was sealed with the membrane in a hybridization bag or assembly (SureSeal, Hybaid, Middlesex, UK) attached to a weight and incubated at 95°C for 2 min (could be omitted) and then at 68°C for 12 hours. The membrane was then washed twice (5 min each) with 2× SSC containing 0.1% SDS at room temperature followed by three washes with 0.2× SSC containing 0.1% SDS at 65 °C for 20 min each, and one wash (5 min) with 0.3% BSA in 1x TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl ) buffer. The membrane was then put in a solution contained 2500x diluted streptavidin-β-AP, 4 % polyethylene glycol 8000, and TBS buffer with 0.3 % BSA,
incubated for 2 h. It was subsequently washed twice (10 min each) with 1x TBS buffer, developed with NBT/BCIP solution. Biotinylated cDNA showed a purple color.

結果(Results)

Gene expression profiles

Using the cluster software (Stanford) to analyze the results from microarray analysis, we initially analyzed the levels of gene expression in three groups of tumor tissues, i.e. meningioma, astrocytoma and GBM, and compared with those of normal brain tissues obtained from patients received an epilepsy surgery. Of the 384 genes, 22 genes had at least two folds increase in their expressions than the mean intensity (Table 1). The results showed that different types of tissues had different gene expression profiles. We found a number of genes that were overexpressed (Nidogen 2, Calreticulin, CRMP3, Chaperonin, TIMP1 and CD44) or underexpressed in GBM compared with normal tissues (Table 1).

We analyzed the data by the hieratical software, and found that these tissues could be departed into two major groups clearly, glioma and meningioma groups (Fig. 1). It also showed the much closer relationship of gene profiles between normal and meningioma tissues. It was correlated to the pathological observations that meningioma was less mitosis and could explain why the meningioma has been diagnosed into a non-malignant tumor and was much easily cured than glioma.
Table 1. The ratio of gene expression levels between different tissue types

<table>
<thead>
<tr>
<th>Gene</th>
<th>M/N</th>
<th>I/N</th>
<th>II-III/N</th>
<th>IV/N</th>
<th>IV/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>0.25473</td>
<td>0.1053</td>
<td>0.163253</td>
<td>0.285852</td>
<td>0.031123</td>
</tr>
<tr>
<td>collagen, type III, alpha 1</td>
<td>0.1523</td>
<td>0.195</td>
<td>0.052</td>
<td>0.036479</td>
<td>-0.11582</td>
</tr>
<tr>
<td>CD44 antigen</td>
<td>0.218424</td>
<td>0.236</td>
<td>0.237883</td>
<td>0.37478</td>
<td>0.156356</td>
</tr>
<tr>
<td>matrix Gla protein</td>
<td>-0.13402</td>
<td>-0.15832</td>
<td>0.041687</td>
<td>-0.0079</td>
<td>0.126128</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>0.163439</td>
<td>0.190443</td>
<td>0.228546</td>
<td>0.271113</td>
<td>0.107674</td>
</tr>
<tr>
<td>EphB3</td>
<td>-0.24074</td>
<td>-0.02726</td>
<td>-0.09538</td>
<td>-0.09037</td>
<td>0.150372</td>
</tr>
<tr>
<td>thrombospondin 4</td>
<td>-0.25111</td>
<td>-0.02795</td>
<td>-0.27132</td>
<td>-0.27132</td>
<td>-0.02022</td>
</tr>
<tr>
<td>protein disulfide isomerase-related protein</td>
<td>-0.37887</td>
<td>-0.14123</td>
<td>-0.25817</td>
<td>-0.26525</td>
<td>0.113617</td>
</tr>
<tr>
<td>MCF.2 cell line derived transforming sequence-like</td>
<td>-0.44843</td>
<td>-0.42554</td>
<td>-0.40841</td>
<td>-0.45107</td>
<td>-0.00264</td>
</tr>
<tr>
<td>platelet factor 4</td>
<td>-0.18781</td>
<td>-0.25382</td>
<td>-0.17339</td>
<td>-0.13086</td>
<td>0.056943</td>
</tr>
<tr>
<td>intercellular adhesion molecule 1 (CD54),</td>
<td>-0.25879</td>
<td>-0.23959</td>
<td>-0.28348</td>
<td>-0.26984</td>
<td>-0.01105</td>
</tr>
<tr>
<td>Laminin alpha 4</td>
<td>-0.43561</td>
<td>-0.45227</td>
<td>-0.37386</td>
<td>-0.42552</td>
<td>0.010087</td>
</tr>
<tr>
<td>Nidogen 2</td>
<td>0.147278</td>
<td>0.10938</td>
<td>0.162346</td>
<td>0.291881</td>
<td>0.144603</td>
</tr>
<tr>
<td>DKKFZP434C211 protein,TEM5</td>
<td>-0.2701</td>
<td>-0.28977</td>
<td>-0.28522</td>
<td>-0.27246</td>
<td>-0.00236</td>
</tr>
<tr>
<td>MMP_2</td>
<td>-0.1269</td>
<td>-0.22146</td>
<td>-0.21484</td>
<td>-0.21293</td>
<td>-0.08603</td>
</tr>
<tr>
<td>interferon induced transmembrane protein 1 (9-27)</td>
<td>0.006454</td>
<td>-0.16511</td>
<td>-0.15275</td>
<td>-0.16074</td>
<td>-0.16719</td>
</tr>
<tr>
<td>IG kappa chain interon</td>
<td>-0.35341</td>
<td>-0.0766</td>
<td>-0.38031</td>
<td>-0.35873</td>
<td>-0.00531</td>
</tr>
<tr>
<td>CRMP2</td>
<td>-0.32263</td>
<td>-0.37121</td>
<td>-0.36593</td>
<td>-0.36566</td>
<td>-0.04303</td>
</tr>
<tr>
<td>CD7 antigen (p41)</td>
<td>0.162294</td>
<td>-0.05088</td>
<td>0.100578</td>
<td>0.095328</td>
<td>0.06697</td>
</tr>
<tr>
<td>MNRP</td>
<td>-0.26313</td>
<td>-0.28146</td>
<td>-0.28146</td>
<td>-0.28146</td>
<td>-0.01833</td>
</tr>
<tr>
<td>eukaryotic translation initiation factor 2B, subunit 5</td>
<td>0.248102</td>
<td>0.227907</td>
<td>0.342858</td>
<td>0.411656</td>
<td>0.163554</td>
</tr>
<tr>
<td>CRMP3</td>
<td>0.151226</td>
<td>0.275117</td>
<td>0.276214</td>
<td>0.32245</td>
<td>0.171224</td>
</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>0.323311</td>
<td>0.004</td>
<td>0.167945</td>
<td>0.29549</td>
<td>-0.02782</td>
</tr>
<tr>
<td>leukotriene C4 synthase</td>
<td>-0.32948</td>
<td>-0.33314</td>
<td>-0.33314</td>
<td>-0.33314</td>
<td>-0.00366</td>
</tr>
<tr>
<td>interleukin 12A</td>
<td>-0.37743</td>
<td>-0.14448</td>
<td>-0.33222</td>
<td>-0.36825</td>
<td>0.009187</td>
</tr>
<tr>
<td>matrix metalloproteinase 12</td>
<td>-0.33557</td>
<td>-0.33557</td>
<td>-0.33557</td>
<td>-0.33557</td>
<td>0</td>
</tr>
<tr>
<td>basic FGF</td>
<td>-0.0655</td>
<td>0.1172</td>
<td>0.020515</td>
<td>-0.06886</td>
<td>-0.00336</td>
</tr>
<tr>
<td>Chapreonin</td>
<td>0.204993</td>
<td>0.20684</td>
<td>0.310112</td>
<td>0.383134</td>
<td>0.178141</td>
</tr>
</tbody>
</table>

The gene expression levels were logtransformated and normalized. N: normal, M: meningioma, I: grade I astrocytoma, II~III: grade II~III astrocytoma, IV: grade IV glioma (GBM).
Fig. 1. A: The dendrograms of the samples clustered based on the 22 genes. B: The six genes: Nidogen 2, Calreticulin, CRMP3, Chaperonin, EIF2B and CD44 were over expression in the glioma group but not in non-malignant tissues. The expression of TIMP1 and EGFR were higher in meningiomas than in gliomas.
We also classified the glioma tissues (grade I ~ grade IV) and compared the gene expression profiles of the different grade glioma tissues with the normal brain tissue (Fig.2). Some genes like Chaperonin, EIF2B, CRMP3, and CRMP1 showed obviously high expression levels in high grade gliomas and low expression levels in normal and low grade gliomas. Another group of genes (IL2, Homo sapiens and BAC clone RP11-791G15) showed an opposite expression patterns (Fig.3). It also showed that the gene expression profiles of low grade glioma were more similar to normal tissues than high grade gliomas. The gene expression profile of highly malignant gliomas was more distant away from normal tissue as the degrees of malignancy increased. These differential gene profiles determined by DNA microarray might provide a valuable tool for the diagnosis of malignant grades in gliomas.

Fig. 2 A. The centroids organized map of different grades of glioma tissues. Each spot represents a tissue specimen, and the order from left to right is normal tissues, low grade gliomas, and high grade gliomas. B. The group which showed a tendency of increasing gene expressions as the degree of malignancy increased contained five genes: CRMP1, CRMP2, eukaryotic translation initiation factor, CRMP3, and Chaperonin.
Fig.3 The dendrogram of gene expressions in different grades of tumors. Five genes (Chaperonin, TIMP1, CRMP3 and CRMP1) were highly expressed in high grade gliomas, and two genes (IL2, Homo sapiens BAC clone) were less expressed.

In addition to the tumor specimens, we also analyzed gene expression profiles in four cell lines (CH-157MN, U-251MG, U-105MG and U-373MG), and compared the results with those from tissues. CH-157MN is a meningioma cell line while U-251MG, U-105MG, and U-373MG are malignant glioma cell lines. After statistical and clustered analysis, we found the gene expression profiles of cell lines differed from those of tumor tissues. They were divided into two major groups (Fig. 4). Some genes were poorly expressed in cell lines (compared to tissues), such as Serum deprivation response, Thy-1 cell surface antigen, CRMP5, CRMP2, Adenomatosis polyposis coli, and LIM domain binding2 etc. The gene expression profiles of clinical tissues might be complicated by the environmental factors (paracrine effects, surgical procedures, …etc.). It is worth noting that cells are different from tissues, and some genes are poorly expressed in cultured cells.
Quantitative PCR

To confirm the RNA expression data obtained by cDNA expression array, we performed Q-PCR for 8 genes which had significant different expression levels. The results showed that the expressions of these 8 genes detected by Q-PCR had the same trend as the results obtained by cDNA microarray. (Fig.4). TIMP1, ID3, nidogen2, breast cancer, LIM domain, chaperonin and PIGF were over expressed in high grade malignant glioma. Steroid was down expressed in highly malignant gliomas.
討論 (Discussion)

Genes overexpressed in GBM

We found a number of genes that were overexpressed in GBM such as Nidogen 2, Calreticulin, CRMP3, Chaperonin, TIMP1 and CD44 etc. Nidogen-2 is a recently discovered extracellular protein that has a length of ~50 nm, binds to collagen IV and perlecan, and is structurally and functionally related to the ubiquitous basement membrane protein nidogen-1 [29]. Dooley et al. showed that the ECM associated protein nidogen-2 appears to be strongly down regulated in the melanoma cell lines by using qRT-PCR [30]. In this study, we showed that nidogen-2 was upregulated in GMB, the significance of this finding is not known at present. Calreticulin is a component of the nuclear matrix. The formation and/or expansion of the calreticulin-nuclear matrix may be related to the activated cell growth [31]. However, controversial results were obtained in another group of researchers who demonstrated that calreticulin selectively inhibited endothelial cell proliferation in vitro and suppressed angiogenesis in vivo [32]. Molecular chaperones comprise several highly conserved families of related proteins, many of which are also heat shock proteins [33]. Its function is still not well understood. Kato et al. showed that primary and metastatic tumors of the brain produce srp 60 and srp may coexpress the other five srps. Upregulation of tissue inhibitor of metalloproteinase (TIMP1) in pancreatic adenocarcinomas has been shown in clinical series [34]. Metalloproteinases are central to matrix degradation and remodelling, which are key events in tumour invasion and metastasis. MMP-2, MMP-9, TIMP-1, and TIMP-2 proteins and activities were noted to be increased in tumors cells of papillary thyroid carcinomas, implied that they might play an important role in the invasion and metastasis of papillary thyroid carcinomas [35]. CD44 is a cell membrane protein often overexpressed on tumor cells and, being both a cell-cell and cell-extracellular matrix adhesion protein, is a determinant factor in cell migration and invasion [36]. GBM rarely metastasizes, but is a highly invasive tumor. CD44 may play an important role in the invasion or migration of glioma cells.

Genes underexpressed in GBM

We found MCF.2 cell line derived transforming sequence-like, Laminin alpha 4 and CRMP2 had a tendency of decreasing as the malignancy of tumor increased. In this study, we found that CRMP1 and CRMP3 were increased but CRMP2 was decreased in GBM. CRMP gene family (collapsin response mediator protein), invasion-suppressor gene, was involved in cancer invasion and metastasis. A low expression of CRMP-1 mRNA in lung cancer tissue was significantly associated with advanced disease, lymph node metastasis, early post-operative relapse, and shorter survival [37]. We could not explain the discrepancy between lung cancer tissue and glioma tissue currently.

Eukaryotic cells express a family of eukaryotic translation initiation factor 2 alpha
(eIF2alpha) kinases that are individually activated in response to distinct types of environmental stress. Also, there are studies showed that eIF2alpha phosphorylation could be involved in the molecular signaling events leading to neuronal apoptosis and death and could be a new target in neuroprotection. Besides, childhood ataxia with central hypomyelination (CACH)/vanishing white matter (VWM) leukoencephalopathy is related to mutations in all five genes of the eukaryotic translation initiation factor (eIF2B) [38].

Compared with GBMs, genes that encode proteins involved in cell motility were expressed at a reduced level in low grade tumors. Moreover, genes involved in the suppression of migration were expressed at higher levels in grade II than in grade IV tumors. The genes overexpressed in high grade gliomas have putative functions in a variety of cellular processes. They can be classified into three major categories: (a) cell growth and transformation, cell cycle control, and apoptosis (MCF.2 cell line derived transforming sequence-like, Calreticulin, EGFR, PDGFR) (b) cytokine, protein kinase, signal transduction and cell surface receptors, and associated proteins (interleukin 12A, IL2, CRMP) (c) cell adhesion, basement membrane and ECM proteins (TIMP3, Nidogen 2, Laminin alpha 4, collagen, type III, alpha 1). It is notable that this study has identified several genes, such as CD44, EGFR MMP2 for which over-expression or down-expression has been previously reported in brain tumors [39], demonstrating the suitability and power of cDNA array technology in the identification of transformation-associated genes [8]. In summary, the establishment of gene expression profiles in brain tumor using cDNA array technology has demonstrated significant expression changes in a number of genes implicated in various cellular pathways related to the control of cell growth, differentiation, and tumor invasion. It provides information for additional molecular studies aimed at a clarification of their role in the development of brain tumors.
27. Shalon D, Smith SJ, Brown PO. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Research


In the beginning of this study, we planned to detect the expression of angiogenic inducers (VEGF, EGF, bFGF, PDGF, TGF-α, TNF-α etc.), angiogenic inhibitors (Thrombospondin-1, Ang-1, Ang-2, angiostatin, TGF-β, etc.), and angiogenic related receptors (VEGFR-1, VEGFR-2, EGFR, and FGFR etc.) in surgical specimens of gliomas by a quantitative reverse transcription polymerase chain reaction (RT-PCR) on mRNA level using a Perkin-Elmer ABI PRISM 7700 sequence detector or with northern blot if necessary. However, the regulation of angiogenesis is too complicated, and the factors involved or related with angiogenesis are so many. Moreover, the relationship between these factors are not clear and their functions are usually redundant. The reviewer of this project suggested us to use microarray for this study. Therefore, we tried to develop a home-made microarray for this study. The membrane contains 384 genes, mostly related with angiogenesis. We then established the gene expression profiles in brain tumors using these cDNA array technology. We demonstrated significant expression changes in a number of genes implicated in various cellular pathways related to the control of cell growth, differentiation, and tumor invasion. It provides information for additional molecular studies aimed at a clarification of their role in the development of brain tumors. We also understand that the cDNA microarray membrane contained 384 genes is not enough for further study, we are currently trying to establish another membrane contained more genes for future studies. However, the reproducibility of the microarray technology is not good enough currently and needs the reconfirmation of data by quantitative real-time PCR.