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Relationship between Apoptosis and Angiogenesis in Nasopharyngeal Carcinoma*

鼻咽癌细胞凋亡与血管生成的关系

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Abstract: To clarify the relationship among apoptosis, angiogenesis, and clinical stage of nasopharyngeal carcinoma (NPC), the apoptosis and intratumoral microvessel density (MVD) in NPC specimens were measured using terminal Deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) method and S-P immunohistichemistry method. The normal nasopharyngeal mucosa (20 cases) and NPC tissues (73 cases including 24 recurrent metastatic cases and 49 non-recurrent cases) were used. The apoptotic rate (AR, number of apoptotic cells/total number of tumor cells) was significantly decreased in NPC than that in normal tissue ($P < 0.01$). MVD and vascular endothelial growth factor (VEGF) expression in recurrent NPC (R-NPC) were higher than those in non-recurrent NPC (NR-NPC) ($P < 0.05$), while AR were lower in R-NPC than in NR-NPC. Expression of VEGF is significantly correlated with MVD, and AR with VEGF ($P < 0.05$). It is revealed that apoptosis might be inhibited in the process of nasopharyngeal carcinogenesis. The expression of VEGF might be involved in angiogenesis of NPC, which may influence the prognosis of the patients. Angiogenesis and apoptosis would be used as parameters to predict the prognosis of NPC patients.

Key words: nasopharyngeal carcinoma, apoptosis, TUNEL, angiogenesis, vascular endothelial growth factor

摘要: 为了探讨鼻咽癌(NPC)细胞凋亡及瘤内血管生成与患者预后的关系, 采用 TdT 酶介导的生物素化 dUTP 缺口末端标记技术(TUNEL)和免疫组化 S-P 法, 分别检测 20 例正常鼻咽粘膜组织及 73 例 NPC 组织的细胞凋亡率(Apoptosis Rate AR)、瘤内微血管密度(MVD)及血管内皮细胞生长因子(VEGF)的表达。其中, NPC 复发转移组 24 例, 未复发组 49 例。结果表明, 正常鼻咽粘膜 AR 显著高于 NPC ($P < 0.01$), NPC 复发转移组的 MVD 及 VEGF 显著高于未复发组 ($P < 0.05$), 而 AR 则显著低于未复发组 ($P < 0.05$), MVD 与 VEGF、MVD 与 AR 呈正相关 ($P < 0.05$)。在 NPC 发展过程中, 细胞凋亡明显受到抑制, VEGF 是血管生成的重要因子, 并通过促进血管生成影响患者的预后, 说明细胞凋亡与 NPC 患者的预后有关系。

关键词: 鼻咽癌 凋亡 TUNEL 血管生成 血管生成因子
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Although nasopharyngeal carcinoma (NPC) is well known as a highly malignant tumor that can invade local tissues and metastasize easily, the mechanism for tumorigenesis of NPC has not yet been clarified. We previously reported that NPC secreted urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor (PAI-

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1)[1,2]. Therefore, degradation of extracellular matrices by u-PA allows NPC tumor cells to invade into local tissue and metastasize. We also demonstrated that high expression of VEGF in NPC stimulated angiogenesis, thereby developing NPC[3].

Apoptosis is a type of cell death, which plays an important role in the homeostasis and development of all tissues within an organism. In contrast to necrosis, apoptosis is a well-regulated physiological process. Any disturbance of the balance between cell proliferation and cell death maintained by apoptosis can result in serious disease, in particular cancer[4]. However, the apoptosis in NPC has not been investigated. In the present study, in order to clarify the relationship among apoptosis, angiogenesis, and clinical stage of NPC, we measured the apoptosis and MVD in NPC specimens using terminal Deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) method and S-P immunohistichemistry method.

1 Materials and methods

1.1 Tissue samples

From 1995 to 1996, biopsy tissue specimens were obtained from 73 patients with NPC at the Department of Otorhinolaryngology of the First Affiliated Hospital of Guangxi Medical University in China. All the specimens were fixed by formalin with neutral pH and embedded in paraffin. Of the 73 cases, 54 were men and 19 were women with the average age of 42 years ranging from 17 to 73 years old. During 3-years follow-up, 19 patients were recurrent and died due to metastasis, 5 patients were not able to follow-up. For comparison, 20 cases of chronic inflammatory nasal mucous membranes were measured.

1.2 Materials

In Situ Cell Death Detection Kit was obtained from Roche Diagnostics GmbH (Germany). Rabbit antihuman factor Ⅷ-related antigen (F8-RA) antibody (ZA-0111), rabbit antihuman VEGF antibody (SC-507), and an S-P immunohistochemistry reagent kit were purchased from Beijing Tyuzan Konsu (Beijing, China).

1.3 TUNEL

To measure the apoptotic cells in NPC tissue, TUNEL method was carried out following manufacture's instruction. In brief, the sections on slides were fixed in 4% formalin/phosphate buffered saline (PBS) for 10 min at room temperature (RT). After rinsed with PBS, the specimens were incubated with 0.1% Triton/ 0.1% sodium citrate for 2 min on ice. All slides except positive control ones were rinsed twice with PBS. Positive control slides were treated with DNase I solution (100ul of 200 ug/ml) for 10 min at RT and rinsed twice with PBS in a separate container, and then combined with other slides. The specimens were incubated with TUNEL reaction mixture for 60 min at 37 ℃. After washed three times with PBS, the specimens were incubated with alkaliphophatase (AP)-conjugated anti-digoxigenin (DIG) antibody solution for 30 min at 37 ℃. For negative control, the sepecimens were incubated with AP-conjugated anti-DIG antibody by omitting the step of TUNEL reaction. After washed with PBS three times, the specimens were equilibrated in 100mM Tris buffer (pH value 8.2) for 5 min at RT, and then substrate solution (flash red substrate) was added to develop colour.

1.4 Immunohistochemistry

Immunohistochemistry was performed as previously described[3,5]. The formalin-fixed, paraffin-embedded specimens were cut into 4-um thick sections and were laid on poly-L-lysine-coated slides. Deparaffinized sections were treated with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activity. For antigen unmasking, the sections were placed in a container and covered with 10 mM sodium citrate (pH value 6.0), and then were heated under microwave at 95 ℃ for 5 min. For staining with F8-RA antibody, the section were further treated by digestion with pancreatin. The sections were incubated with normal goat serum for 20 min and incubated at 4 ℃ overnight with rabbit-human F8-RA antibody (ZA-0111), with rabbit antihuman VEGF antibody (SC-507), or with normal rabbit serum serving as a negative control. The sections were then washed with phosphate-buffered saline (PBS, pH value 7.2) and incubated for 20 min with biotinylated goat anti-rabbit immunoglobulins at 37 ℃. After washed three times with PBS, the sections were incubated for 20 min with avidin-biotin peroxidase complex reagent at RT. Immunocomplex was visualized by adding of synthesized substrate, 3' 3-diaminobenzidine tetrachloride dissolved in 0.03% hydrogen peroxide. The sections were counterstained with hematoxylin and mounted.

1.5 Statistical analysis

The Mann-Whitney U test was used to determine the relationship among the expression of VEGF, MVD, AR, and clinical stage of NPC. Correlations between the expression of VEGF and MVD or AR were examined by Spearman rank

correlation coefficient using PEMS, statistic software developed by the Department of Statistics, Huaxi College of Medicine in China.

2 Results

For evaluation of TUNEL, the cells were assessed for morphology characteristic of apoptosis and stained according to Gold et al[6]. The cells with red coloured condensation of chromatin and cytoplasm were referred as apoptotic cells (Fig. 1). AR value was obtained by the number of apoptotic cells out of serially counted 1000 cells. The cells positively expressing VEGF were judged by their cytoplasm stained brown by SC-570 antibody (Fig. 2). Apoptotic cells and VEGF-positive cells have been found in the test of TUNEL and immunohistochemistry of serially sliced sections from the same specimen of NPC. And VEGF was not only expressed in NPC cells, but also in stromal lymphocytes. Vascular Endothelial cells were stained brown by F8-RA antibodies (Fig. 3). The definition of microvessels and calculation of MVD were performed as previously reported. The microvessels in NPC were unequally distributed, which were rich in the surrounding connective tissue around tumor cells

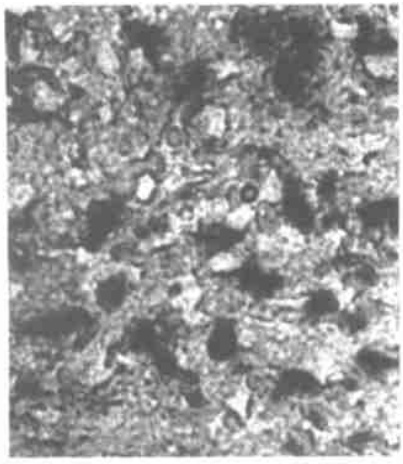

Fig. 1 Apoptosis cells stained by flash red which were scattered in nasopharyngeal carcinoma(TUNEL× 400).

and were rare in tumor. The expression of VEGF and MVD in NPC were significantly higher than those in normal nasal mucous membrane (Table 1).

Table 1 Comparison of apoptotic rate(AR), micrivessel density (MVD), and the expression of vascular endothelial growth factor (VEGF) between normal pharyngeal mucosa (PM) and nasopharyngeal carcinoma (NPC)

Group	No of cases	AR	MVD	VEGF
PM	20	3.17±1.63	2.63±0.96	0.33±0.48
NPC	73	1.72±1.21 *	14.58±4.78 *	1.95±0.79 *

Data is represented as mean±standard deviation. * $P<0.01$ (NPC vs. PM).

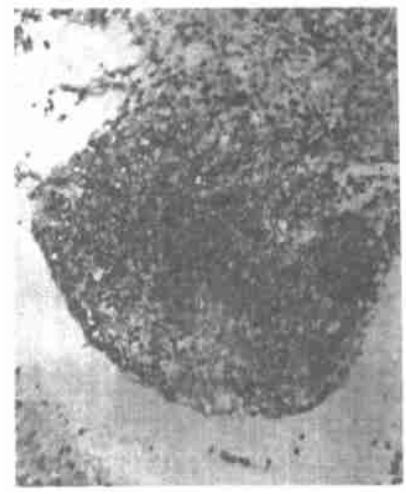

Fig. 2 Vascular endothelial growth factor positive cytoplasma and nuclear of nasopharyngeal carcinoma, which were stained as brown-yellow using rabbit anti-human VEGF(S-P× 400).

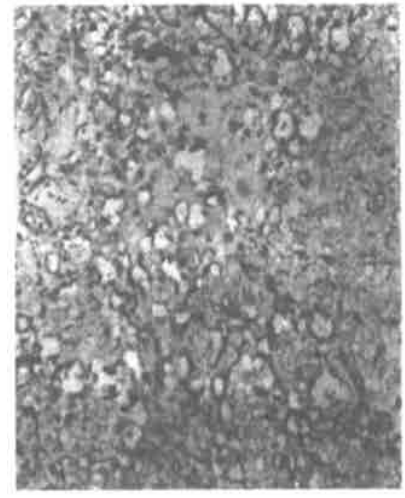

Fig. 3 Microvessels distributed in interstitial area of NPC(S-P × 200).

Although few apoptotic cells were observed in NPC, the AR of normal tissue was significantly higher than that of NPC ($P < 0.01$). The AR of recurrent metastatic NPC (R-NPC, 19 cases) was significantly lower than that of non-recurrent metastatic NPC (NR-NPC) ($P < 0.05$). There was a close correlation between both the expressions of VEGF and MVD, which significantly increased as the stage of NPC progressed ($P < 0.05$, advanced stage vs. early stage). Moreover, the expression of VEGF and MVD in R-NPC were significantly higher than those in NR-NPC ($P < 0.05$). In the analysis of correlation coefficients of AR, MVD, and the expression of VEGF, the expression of VEGF is significantly correlated with MVD ($r = 0.73$, $P < 0.01$) and AR ($r = 0.45$, $P < 0.05$).

3 Discussion

In the present study we demonstrated that the expres-

sions of VEGF, MVD, and AR were correlated with the development and metastasis of NPC. During 3-years follow-up, the expressions of VEGF and MVD in 23 cases those died from recurrent metastatic NPC (R-NPC) were higher than those in non-recurrent metastatic cases, while AR was significantly lower in R-NPC.

Many cancers occur due to either normal apoptosis in combination with enhanced cell proliferation or normal cell proliferation with down-regulated apoptosis. We have shown that the expression of p16, one of the tumor suppressor gene products, was down-regulated in NPC, indicating that progression of cell cycle might be enhanced[5]. Furthermore, Epstein-Barr Virus (EBV) is known to be a strong candidate for the cause of NPC[7]. Zhao et al demonstrated that aberrant overexpression of the cyclin D1 protein in NPC cell lines, the expression of which might be increased by latent membrane protein 1 (LMP1) encoded by EBV[8]. Taken together, the cell proliferation of NPC is enhanced.

Our study demonstrated that AR was significantly lower in NPC tissue than in normal nasal mucous membrane. It is well known that apoptosis can be induced by various causes such as 1) activation of plasma membrane receptors for tumor necrosis factor (TNF)α, Fas, and NMDA, 2) activation of p53 due to DNA damage, 3) stress (e.g., growth factor withdrawn, reactive oxygen species), and 4) increase in cytosolic Ca^{2+}. Since there was also infiltration of stromal lymphocytes surrounding NPC tumor cells, we first considered that apoptosis might be induced by cytotoxic T cells (CTL). CTL can kill the cancer cells by producing Fas ligand (FasL) on their surface, which binds with the Fas on the target cells leading to its death by apoptosis. However, many apoptotic cells were seen when NPC cells were implanted into immunodefficient nude mouse[9]. It is suggested that apoptosis in NPC tissue might not be induced by cellular immunity.

Some viruses can prevent apoptosis of the cells they have transformed. EBV can produce a protein similar to Bcl-2 or can produce another protein that stimulates the production of Bcl-2[10]. Thus, the cells become more resistant to apoptosis, thereby enabling the cancer to proliferate continually. Some cancer cells can even produce Bcl-2 without the participation of viruses. For example, some B-cell leukemias and lymphomas express high level of Bcl-2, thus blocking apoptotic signals they may receive[11]. Melanoma cells avoid apoptosis by inhibiting the expression of the gene encoding Apaf-1[12].

Immunohistochemistry of serially sliced sections showed that NPC cells surrounding apoptotic cells highly expressed VEGF. This can be interpreted by following reason. Namely, relative low level feeding to tumor cells due to delay of angiogenesis induces hypoxia at local tissue of NPC, which thereby induces up-regulation of VEGF expression with the resultant angiogenesis or stimulates apoptosis of tumor cells. Our previous study and the present study demonstrated that the expressions of VEGF and MVD were significantly increased in NPC (especially in advanced stage). Moreover, the expression of VEGF is correlated with MVD, suggesting that the angiogenesis in NPC might be regulated by the expression of VEGF in NPC.

However, in some cases of NPC tissue, the down-regulated apoptosis was found despite high-level expression of VEGF. This implicates that the tumor cells may acquire the ability to survive under hypoxia condition. These tumor cells, which are resistant to hypoxia, might be the reason for difficulty of treatment and for recurrent and metastasis of NPC. Therefore, although the apoptosis of NPC cells can be induced by hypoxia in early stage, the tumor cells might be able to further transform into more proliferate cells by acquiring the ability to block the apoptosis pathways.

4 Conclusion

The cases with high AR values were sensitive to cancer therapies, thereby the survival ratio was significantly high. This implicates that the measurement of AR would be a useful parameter for the evaluation of treatment and the prognosis of patients. Therefore, they can be measured as informative parameters for prognosis of patients with NPC.

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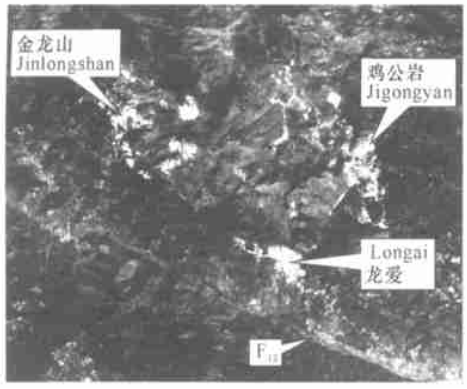

图 5　研究区 QuickBird-2 遥感影像

Fig. 5　The QuickBird-2 photo of remote sensing in study area

趋势的高值区与研究区南部的深大断裂(F_{12})的位置十分吻合。因此, 可以尝试将高分辨率遥感线性构造作为深部构造解译的重要参数。

4　结论

(1)应用盒计维数法, 在 2.000～0.125km 的标度范围内, 经过统计计算求得高龙金矿及其外围的分维值 $D = 1.660$, 相关系数 $R = 0.999$, 表明研究区线性构造具有分形特征, 其分形结构具有很好的统计自相似性。

(2)研究区内已知的 3 个矿段均处于分维高值区内, 说明分维值越高, 成矿的可能性越大。

(3)就整个矿区而言, NWW 向线性构造的分维值大于近 SN 向线性构造的分维值, 说明研究区内 NWW 向的线性构造较近 SN 向线性构造更为发育。

(4)遥感线性构造的分维能够反映地壳深部的构造信息, 而深部构造又是矿区成矿的重要因素, 因此可以尝试将高分辨率遥感线性构造作为成矿区预测和深部构造解译的重要参数。

致谢

在对QuickBird-2 高分辨率遥感资料进行解译的过程中得到桂林工学院吴虹研究员的热心指导, 在此深表谢意。

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