行政院國家科學委員會專題研究計畫成果報告

非小細胞肺癌之第12型母質金屬蛋白酶與血管生成

Matrix metalloproteinase-12 and angiogenesis in non-small cell lung cancer

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計畫中文摘要

癌的組織侵犯過程是由多重步驟造成，包括蛋白質融解及癌細胞與間質細胞之間的互動。母質金屬蛋白酶(簡稱 MMP)為一群可融解細胞外母質之融解酶家族，其功能為主導生物發育過程，炎症反應及許多病程 (包括癌之侵犯、血管生成及轉移)之組織重建與修複。多種細胞均可分泌 MMP，包括癌細胞，炎症細胞及各型間質細胞。而癌組織中常同時表現多種 MMP，而這些 MMP 之表現，在過去的研究中，被證實與腫瘤之預後或期別有關。

人類巨噬細胞彈性纖維融解酶(HME，又稱 MMP-12)，是由人類巨噬細胞所分離出來，可融解許多母質蛋白，並與 angiostatin 之生成有關。Angiostatin 被認為可抑制血管生成，而後者又為癌症轉移及預後之重要因子。因此，可合理推論，MMP-12 之表現與癌症之預後可能有關，此點在肝癌已被證實。但至今並無任何研究探討 MMP-12 與肺癌之臨床表現與預後之相關聯性。

本研究計劃之目的為探討：【1】MMP-12 表現在肺癌中之發生率，與分佈特性，【2】肺癌組織中表現 MMP-12 之細胞種類，【3】與肺癌組織中血管生成密度之相關聯性，及【4】MMP-12 表現與癌症臨床期別及其預後之關係。關鍵詞：母質金屬蛋白融解酶，人類巨噬細胞彈性纖維融解酶、血管生成、肺癌

計畫英文摘要

Tumor invasion is a multistage process in which cellular motility is associated with controlled proteolysis and involves interactions between tumor cells and the extracellular matrix. The matrix metalloproteinases (MMP) are a family of matrix degrading enzymes that are important in tissue remodeling and repair during development, inflammation, and various diseases process, including tumor invasion, angiogenesis and metastasis. Many kinds of cells can express MMPs, including tumor cells, inflammatory cells, and stromal cells. The expression of certain MMPs may play important roles in tumor stage and prognosis.

Human macrophage elastase (HME, MMP-12) is a MMP that was initially found in alveoalr macrophage. HME can degrade many matrix substrates, and is related to the production of angiostatin. Angiostatin can inhibit angiogenesis, while the latter is a well-recognized prognostic factor for many tumor types. Therefore, a reasonable deduction is that the expression of HME is a prognostic marker of cancer, which has recently been demonstrated in hepatocellular carcinoma.

In this study, we intend to apply sizable number of clinical specimens to study: [1] the rate of HME mRNA expression in lung cancer; [2] the identification of major cell types expressing HME; [3] the correlation of microvessel counts, angiostatin production and HME expression; and [4] the implication of HME mRNA expression on the survival of patients with lung cancer. Keywords：Matrix metalloproteinase, human macrophage elastase, angiogenesis, lung cancer

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of related zinc-dependent, matrix-degrading enzymes that are important in tissue remodeling and repair during development and inflammation.[1, 2]. The MMP gene family consisted of 20 members, collectively capable of degrading essentially all ECM componets, and can be classified into five groups according to their substrate specificity and structure : (1) Collagenase – MMP-1, -8, -13 ; (2) Stromelysins – MMP-3, -10, -12, -7 ; (3) Gelatinases – MMP-2, -16 ; (4) Membrane-type MMPs – MMP-14 -15, -16, -17, and (5) other MMPs- MMP-11, -19, -20.[1] The activity of MMPs in extracellular space is specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to the highly conserved zinc binding site of active MMPs. The TIMP gene family consists of four members, TIMP-1, -2, -3, and -4. TIMP-1, -2, and -4 are secreted in soluble form, whereas TIMP-3 is associated with extracellular matrix (ECM).

Abnormal expression of MMPs is associated with various diseases such as tumor invasiveness, arthritis, and atherosclerosis. [3] Tumor invasion, metastasis and angiogenesis require controlled degradation of ECM, and increased expression of MMPs.[4] The expression of MMPs in tumors is regulated in a paracrine manner by growth factors and cytokines secreted by tumor infiltrating inflammatory cells as well as by tumor or stromal cells. Recent studies have suggested continuous cross talk between tumor cells, stromal cells, and inflammatory cells during the invasion process. [5,6] Cytokines and growth factors releases by inflammatory cells can induce the expression of MMPs. The MMPs are predominantly produce by surrounding stromal and inflammatory cells. Tumor cell-derived factors that increase the expression of several MMPs in stromal cells have been purified.[7] MMPs themselves are able to degrade and inactivate IL-1β and cleave the TNF-α precursor to a biologically active form, as well as the capacity of TIMP-3 to inhibit activation of TNF-α, indicating that MMPs and TIMPs can regulate inflammatory cytokines at the site of tumor invasion.[8].

Angiogenesis, the formation of new blood microvessels, is an obligatory event connected with tumor growth, invasion and metastasis.[9,10] The level of angiogenesis is a well recognized prognostic indicator in many tumor types. [11, 12] Endothelial cells of microvessel sprouts secrete several extracellular matrix-degrading enzymes, including MMPs (mainly MMP-2 and MMP-9), which allow spread of tumor cells into and through the adjacent matrix.[13] MMPs secreted by other stromal cells and tumor cells also contribute to the lateral expansion of new vessels.

Many reports have already described the role of MMPs in tumor invasion.[14, 15] MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-11 and MMP-14 have been detected in different histologic types of lung cancer by various approaches.[16,17] The expression of the MMPs was essentially from stromal cells, rather than tumor cells. Co-expression of several MMPs was noted, especially in cancer with advanced stage, but no studies could demonstrate the implication of MMP on the survival of patients with lung cancer. [18,19]

Human macrophage metalloelastase (HME, MMP-12) is a MMP that was initially found in alveolar macrophages of cigarette smoker by Shapiro et al. [20] HME has been shown to participate in the degradation of elastic fibers and involves in the pathogenesis of pulmonary emphysema and atherosclerosis. [21,22] More recently, HME was shown to involve in the process of angiostatin formation, which is an internal fragment of plasminogen with an angiogenesis-inhibiting function. [23,24] Angiostatin derived from some murine primary tumors can inhibit the growth of the metastatic foci through the suppression of the neovascularization and increase of apoptosis. Recently, Gorrin Rivas et al. had studied the expression of HME in hepatocellular carcinoma (HCC). HME was mainly expressed in tumor cells, and had a significant association with the production of

angiostatin. Patients with tumors not expressing HME had poorer survival than those with higher levels of HME expression. This implies that HME may serve as a new prognostic marker in HCC.[25]

MATERIALS AND METHODS

Patients and tissues

Sixty-five pairs of surgical specimens of tumors and the adjacent uninvolved lung tissue will be obtained form patients with lung cancer at the time of resection. All were non-small cell lung cancer confirmed by histological diagnosis. After excision, tumor samples and the uninvolved lung tissues were collected immediately, snap frozen and stored at -70°C until processed. Specimens used for paraffin embedding and for OCT embedding are collected separately.

The resected lung and lymph nodes are subjected to routine surgical pathological examination. Representative sections required for staging and histologic classification are generously taken. Sections of 4μm thickness were routinely stained with hematoxylin-eosin. Histological classification is based on World Health Organization criteria. The final staging of each patient was pathologic, according to the international staging system for lung tumors.

RNA extraction and RT-PCR

Total RNA was isolated form tumorous and nontumorous lung tissues by the acid guanidinium-thiocyanate/phenol/chloroform method. Reverse transcriptions are performed using Superscript RTase in a volume of 20 μL. The PCR primer sequences are as follows: MMP-1 (786 bp) sense 5'-CGACTCTAGAA ACACAAGAGCAAGA, antisense 5'-AA GGTTATCTTACTGTCACACGCTT; MMP-2 (580 bp) sense 5'-GTGCTGAAGGACACA CTAAAGAAGA, antisense 5'-TTGCCATCC TTCTCAAAGTTGTAGG; MMP-3 (515 bp) sense 5'-AGATGCTGTTGATTCTGCTGTT GAG, antisense 5'-ACAGCATCAAAGGAC AAAGCAGGAT; MMP-9(243bp) sense 5'-CACTGTCCACCCCTCAGAGC, antisense 5'-GCCACTTGTCGGCGATAAGG; MMP-12 (345 bp) sense 5' –AGCAAGATTAACA CAGGCAT, antisense 5' –GTCTCCATAACA GGGACTGAA; TIMP-1(667 bp) sense 5'AT CCTGTTGTTGCTGTGGCTGATAG, anti-sense 5'-TGCTGGGTGGTAACTCTTTATT TCA; TIMP-2(405 bp) sense 5'-AAACGAC ATTTATGGCAACCCTATC, antisense 5'-A CAGGAGCCGTCACTTCTCTTGATG.

Microvessel Staining and Counting

Microvessels were stained by using mouse polyclonal anti-CD34 antibody (1:20 dilution) (Novocastra, Newcastle, UK) as the primary antibody, with binding visualized through the avidin-biotin-peroxidase-complex method. Any brown-stained endothelial cell separated from adjacent microvessels was considered as representing a single microvessel. The tumor sample was first examined at a low power (×100) to identify the area with the highest density of microvessels, after which all microvessels in three ×200-power fields (×20 objective and ×10 ocular magnification, 0.785 mm^2 per field) in this area were counted, and the average of three readings was taken as the microvessel count (MVC).

RESULTS

Patient demographic data

Of 65 NSCLC patients, most of them received curative resection of cancer (28 were stage I, 15 were stage II, 20 were stage III and 3 were stage IV). The distribution of histology subtypes are: adenocarcinoma 37 cases, squamous cell carcinoma 23 cases, large cell carcinoma 2 cases, spindle cell carcinoma 2 cases, carcinoid 1case.

Expression of MMPs and TIMPs

Tumor tissues commonly expressed higher MMPs and less TIMPs mRNA than their nontumrous counterparts. (overexpression of MMP-1 53%, MMP-2 30%, MMP-9 59%,, MMP-12 83.3%, TIMP-1 31.8%, TIMP-2 33.3%). Correlation of the expresison of MMPs and TIMPs and pathological stage and histologic subtypes was noted as: MMP2 and advanced N stage, TIMP2 and overall pathological stage, especially with T stage.

Expression of MMP-12 and TIMP-1 is

associated with better survival outcome in NSCLC

After a mean follow-up period of an average of 3.5 years, survival analysis favors patients with the expression of higher amount of MMP-12 (P=0.025) and TIMP-1 (p=0.008), while disfavor the expression of MMP-2 (p=0.005).

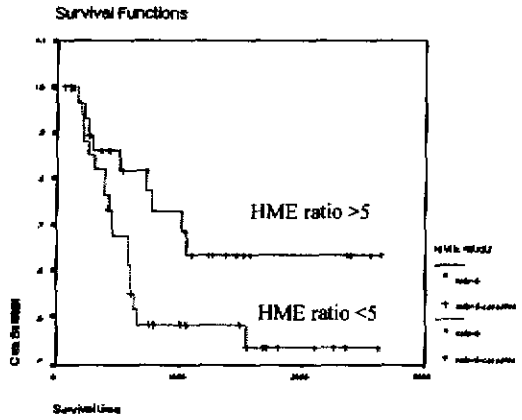

Fig 1. Overall survival and the expression of MMP-12 (HME) in 65 NSCLC patients

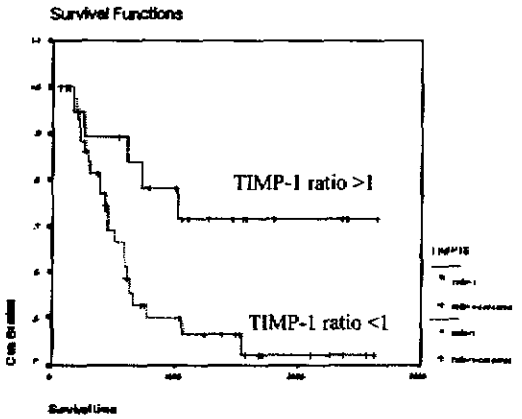

Fig. 2 Overall survival and the expression of TIMP-1.in 65 NSCLC patients

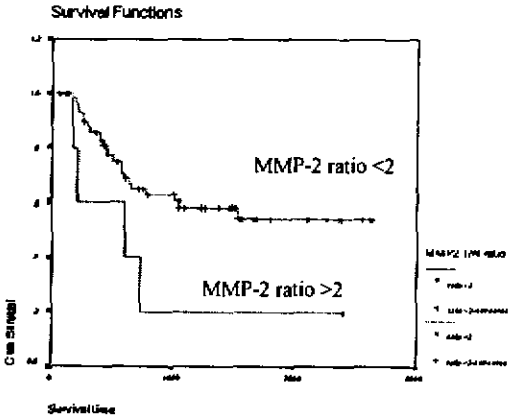

Fig. 3 Overall survival and the expression of MMP-2 in 65 NSCLC patients

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