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放射線活化肝癌細胞侵襲轉移能力的分子生物機轉研究
研究成果報告(精簡版)

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中華民國96年10月16日
放射線活化肝癌細胞侵襲轉移能力的分子生物機轉研究

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執行單位：國立台灣大學醫學院臨床醫學研究所

中華民國 96 年 10 月 7 日
Abstract

This study is to investigate the molecular mechanism of radiation enhanced cell invasiveness of hepatocellular carcinoma (HCC) correlating with clinical patients undergoing radiotherapy and subsequently developing metastasis. Three HCC cell lines (HepG2, Hep3B, and Huh7) and normal hepatocyte cell line (CL-48) were irradiated with different doses. The effect of radiation on cell migration and invasiveness was determined using the Boyden chamber assay. Radiation enhanced migration-invasion capability was evident in HCC cells but not in normal hepatocytes. Invasion-migration was observed in gelatin-coated but not fibronectin-coated or type I collagen-coated membranes. Radiation up-regulated matrix metalloproteinase-9 (MMP-9) mRNA level, MMP-9 protein level, and MMP-9 activity. MMP-9 antisense oligonucleotides inhibited radiation induced MMP-9 expression and thereby significantly inhibited radiation induced HCC invasion-migration. Furthermore, PI3K/Akt chemical inhibitors LY294002 and wortmannin suppressed radiation induced MMP-9 mRNA expression. Transient transfection with dominant negative Akt plasmid also showed that the PI3K/Akt signaling pathway was involved in this radiation induced MMP-9 expression. Moreover, NFκB decoy oligodeoxynucleotide suppressed radiation enhanced MMP-9 promoter activity completely. PI3K/Akt chemical inhibitors inhibited radiation induced NFκB-driven luciferase promoter activity. Taken together, our results indicated that sublethal dose of...
radiation could enhance HCC cell invasiveness and migration by MMP-9 expression through the PI3K/Akt/NFκB signal transduction pathway.

**Keywords:** Radiation, Hepatocellular carcinoma, Invasion, Migration, Signal transduction.

二、計畫緣由與目的

Radiation has been a cancer therapy for more than a hundred years. Nowadays, more than 50-60% of cancer patients need radiotherapy as a part of their cancer treatment. The rationale of radiotherapy in cancer treatment is based on the observation that radiation can inhibit cell proliferation or induce apoptotic cell death *in vitro*, and suppress tumor growth *in vivo*. Besides the therapeutic effect, recent evidence has shown that irradiation may promote malignant behaviors of cancer cells both *in vitro* and *in vivo* by activating several pathways involved in tumor invasiveness and metastasis.

The metastasis of cancer causes the majority of human cancer deaths. It remains the greatest barrier to cancer cure. Cancer cell invasion and metastasis are complicated processes involving several classes of proteins (e.g., calcium-dependent cadherins, integrins, extracellular proteases, angiogenetic factors, and lymphangiogenesis factors). Among these factors, tumor-associated matrix metalloproteinases (MMPs) have been important components of this process because they degrade extracellular matrix proteins. The expression and activity of MMPs increase in almost all human cancer types and are associated with advanced tumor stage and poor survival. Increased plasma level of MMP-9 has been found in lung cancer and breast cancer during radiotherapy. Moreover, an MMP inhibitor has been shown to block radiation-induced invasiveness of human pancreatic cancer cells.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Radiotherapy has been integrated into one of the treatment modalities. Several clinical studies showed that a significant proportion of HCC patients undergoing radiotherapy subsequently develop intrahepatic and extrahepatic metastasis. This development was associated with a detrimental outcome. In this study we were mainly interested in clarifying the molecular mechanism of radiation induced HCC invasiveness, migration, and metastatic capability. We exposed HCC cell lines to sublethal doses of radiation and showed that radiation enhanced invasion-migration capability. The capability was dependent on increased MMP-9 activity mediated mainly through the PI3K/Akt/NF-κB signaling transduction pathway.

三、結果與討論

*Radiation enhances invasiveness of HCC cell lines*

Three human HCC cell lines, HepG2, Huh-7, and Hep3B, and the normal hepatocyte cell line, CL-48, were plated in Boyden chambers with Matrigel-coated membranes, mimicking the basement membrane which serves as a barrier to migration. The cells were irradiated with different doses from 5 Gy to 10 Gy. After 16 hours, the cells were fixed, stained with crystal violet, and counted. The result indicated that 7.5 Gy of radiation significantly enhanced cell migration and invasiveness. The effect was evident for all three HCC cell lines but not the normal hepatocyte cell line. We further confirmed the invasiveness of HepG2 cells by an intravasation CAM assay. HepG2 cells were irradiated with 7.5 Gy before being seeded on the upper CAM. After 48 hours, cells invading the lower CAM were quantified using PCR of the human genome specific Alu sequence. The result indicated that radiation enhanced the invasiveness of HepG2 cells. The radiation-enhanced invasiveness in the CAM assay was evident in all three HCC cells but not in normal hepatocyte cell line.

*Radiation dose response of HCC cells and normal hepatocytes*

The trypan blue exclusion assay showed
reduced growth rate of all cell lines irradiated with 7.5 Gy. However, cell death was rare at this dose either at 24 hours or 48 hours. Cell cycle distribution of HepG2 cells at 24 hours by propidium iodide staining and flow cytometry showed no obvious subG1 cell death peak. The distribution of G1 and G2/M was shifted with G2/M arrest. Results of a colony formation assay confirmed the long-term effect of radiation on HepG2 and Hep3B cells. The morphology and quantification data demonstrated that 7.5 Gy still inhibited HCC cell growth.

Radiation induced HCC invasiveness depends on cell matrix component specificity

Extracellular matrix, which acts as a tissue barrier, has different components such as gelatin, collagen, fibronectin, etc. The invasiveness of cancer cells is frequently associated with their ability to degrade certain of these matrix materials. We were interested in identifying the matrix composition most vulnerable to radiation enhanced invasion by tumor cells. HepG2 cells were seeded on Boyden chamber membranes coated with different cell matrix materials, including Matrigel, gelatin, fibronectin, and type I collagen, and irradiated with 7.5 Gy. After 16 hours, the migrated cells were fixed and stained with crystal violet, and high power fields were digitally photographed. The results show that the radiation enhanced HepG2, Huh7, and Hep3B cells could migrate through gelatin, respectively. The results imply that cell invasiveness correlates with specific enzyme activities and that these are critical in radiation enhanced HCC cell invasiveness.

MMP-9 expression is involved in radiation enhanced HCC cell invasiveness

The cancer cell invasiveness has been linked to increased expression of MMP proteins. Several MMP family proteins, including MMP-1, -2, -3, and -9, are overexpressed and associated with poor prognosis in HCC patients. RT-PCR expression patterns of MMP-1, -2, -3, and -9 mRNA in HepG2 cells showed that MMP-1, -2, and -3 mRNA were down-regulated, but MMP-9 mRNA was upregulated, with increasing radiation dose. However, 10 Gy did not increase MMP-9 mRNA expression, which might be attributed to severe damage of cell protein at this dose. The protein level of cytosolic MMP-9 as well as in the cell culture supernatant and its protein activity by zymography confirmed this expression pattern. The protein level of MMP-9 increased significantly in three HCC cell lines irradiated with 7.5 Gy, but not in normal hepatocytes.

The role of MMP-9 in radiation enhanced HepG2 cell invasiveness was further tested using an anti-sense strategy to inhibit MMP-9 gene expression. HepG2 cells were treated with antisense (As) MMP-9 oligonucleotide or sense (S) oligonucleotide before being irradiated and then assayed for Matrigel invasion capability. Treatment with As MMP-9 oligonucleotide significantly inhibited the radiation enhanced HepG2 cell invasiveness. The effect of As MMP-9 oligonucleotide was confirmed by Western blotting using MMP-9 specific antibody. Similar inhibitory effect of As MMP-9 oligonucleotide on radiation enhanced invasiveness was shown in the in vivo CAM assay. Therefore, the ability of radiation enhanced HCC cells to invade Matrigel-coated membranes is dependent on the induction of MMP-9 expression.

Radiation activated PI3K/Akt signal transduction pathway is involved in radiation enhanced MMP-9 expression

Radiation has been found to activate several intracellular signaling pathway mediators, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K). We investigated the effect of radiation on the downstream activation of the MAPK and PI3K pathways by detecting phosphorylated p38, ERK, JNK, and Akt. Furthermore, to inhibit phosphorylation of these proteins, we used SB203580 for p38, PD98059 for MAPK/ERK, LY294002 for PI3K/Akt, and SP600125 for JNK. Radiation (7.5 Gy) was found to promote p38, ERK, JNK, and Akt phosphorylation in HepG2 cells. By pretreating with different chemical inhibitors and assaying MMP-9 protein levels in cell culture supernatants (by EIA), we showed that radiation induced MMP-9
protein expression in HepG2 cells could be inhibited by the PI3K/Akt specific inhibitor LY294002. We further examined the PI3K/Akt signaling pathway by performing a 72-hour transient transfection of HepG2 cells with dominant negative (dn)Akt to block the phosphorylation of wild-type cytosolic Akt. The transfection efficiency (calculated from green fluorescent protein expression levels) in HepG2 cells using our protocol was about 21%. The ratios of phosphorylated-Akt/nonphosphorylated Akt in dnAkt transfectants and vector controls (pcDNA-3) to that in sham controls measured by densitometry were 1.5 and 1.9, respectively. The phosphorylated-Akt/nonphosphorylated Akt inhibition ratio of the dnAkt transfectants was 21% less than that ratio in pcDNA-3 (vector) controls. In addition, the ratios of MMP-9 in dnAkt transfectants and vector controls to that in sham controls measured by densitometry were 1.3 and 1.7, respectively. The MMP-9 inhibition ratio of the dnAkt transfectants was 23% less that that of the pcDNA-3 group. The transient transfected-dnAkt cell line exhibited a marked reduction in radiation-induced MMP-9 expression when compared with the vector control. The above findings indicate that PI3K and its downstream Akt are activated by radiation and are critically involved in radiation induced MMP-9 expression.

PI3K/Akt signal transduction mediated NF-kB activation is involved in radiation induced MMP-9 expression

The regulation of the radiation effect on MMP-9 was investigated at the transcriptional level using a luciferase reporter plasmid containing a 0.7-kb segment at the 5’-flanking region of the human MMP-9 promoter region. There were NF-kB and AP-1 binding sites on this MMP-9 promoter region. After transfection with MMP-9 promoter luciferase plasmid for 24 hours, HepG2 cells were treated one hour prior to irradiation (7.5 Gy) with a cis element decoy agonist NFk-B binding site (decoy NF-kB), AP-1 binding site (decoy AP-1), or scrambled decoy (SD) to block the transcriptional factor binding site. After 6 hours, the MMP-9 promoter luciferase activity was determined (Figure 6A). Radiation did induce MMP-9 promoter luciferase activity. However, this induction could be significantly suppressed by decoy NF-kB but not by decoy AP-1 or scrambled decoy.

Our next step was to test whether PI3K/Akt signaling is involved in radiation induced NF-kB activity, using a NF-kB binding site driven luciferase activity assay. After transfection with NF-kB binding site driven luciferase plasmid for 24 hours, HepG2 and Hep3B cells were treated for one hour prior to irradiation (7.5 Gy) with PI3K/Akt chemical inhibitors, LY294002 and wortmannin, or DMSO solvent control (vehicle). After one hour, the NF-kB promoter luciferase activity was determined as described in materials and methods. The radiation induced NF-kB promoter luciferase activity was significantly suppressed by both PI3K/Akt chemical inhibitors but not by the DMSO solvent control.

The PI3K/Akt signaling pathway involved in radiation induced NF-kB activity was further examined by detecting NF-kB nuclear translocation. HepG2 cells were treated one hour prior to irradiation (7.5 Gy) with PI3K/Akt chemical inhibitors LY294002. After one hour, the cytosol and nuclear proteins were isolated and assayed for NF-kB p65 by Western blotting with specific antibody. The radiation induced NF-kB p65 nuclear translocation was inhibited by LY294002 and thereby showed PI3K/Akt signaling was involved in this process.

Finally, the involvement of PI3K/Akt signaling and NF-kB in radiation induced MMP-9 expression was confirmed by zymography of MMP-9 enzyme activity. HepG2 cells were treated for one hour prior to irradiation (7.5 Gy) with the PI3K/Akt chemical inhibitor wortmannin, or the NF-kB chemical inhibitor BAY11-7082. After 24 hours, the cell culture supernatant was collected for analysis by zymography. Both wortmannin and BAY11-7082 reduced radiation induced MMP-9 enzyme activity.
和蛋白质水平。

四、計畫成果自評

在这一年资助下，我们成功地提出了信号转导途径，使其成为辐射增强侵袭性的HCC细胞。这些发现支持临床情况下加速转移性的一些HCC患者在放射治疗后的情况。随后的在体动物研究正在进行中。

研究工作中的这一年的资助已经基于预定的计划表。这些结果的项目已经发表在一项SCI论文（Oncogene 2006; 25:7009-7018）。

最初的项目被设计为一个3年研究，包括体外和在体实验。最后被修改为一个1年项目由国家科学委员会。因此，预算限制在第一年为体外实验。初步的在体研究不可用，也无法完成预定的计划表，因此可能需要延迟。然而，分子机制和在体应用结果从整个项目是非常重要，对临床使用放射治疗在HCC患者。希望的后续研究将尽快进行，以帮助阐明辐射对HCC控制和可能的传播。

五、参考文献


出席國際學術會議心得報告

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<td>出國人員姓名</td>
<td>成佳憲 / 國立台灣大學醫學院臨床醫學研究所助理教授</td>
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<td>第 100 屆美國癌症研究協會年會</td>
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<tr>
<td>發表論文題目</td>
<td>MMP-9 involves radiation-enhanced in vitro invasiveness and in vitro pulmonary metastasis of Lewis lung carcinoma.</td>
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一、參加會議經過

在國科會計畫補助下，於 96 年 4 月 14-18 日赴美國加州 Los Angeles 發表研究論文於第 100 屆美國癌症研究協會(American Association for Cancer Research)年會，其間於 4 月 15 日上午 8 時至 12 時為本次發表論文海報展示討論時段，共計有二千餘人次之各國研究學者至本研究展示區，對本研究內容細節詢問與討論，並有連絡資料的相互交換。

此外於大會期間的其他時段，亦參與多項 Symposium，Plenary section，Education section，獲益良多。

二、與會心得

本屆 AACR 年會適逢第 100 屆，故選在交通便利的 Los Angeles 舉行，以利世界各國學者共襄盛舉，也確實吸引了 5000 人以上的參加人員，共同發表討論目前癌症研究的最新發展及前瞻性試驗。在今年的會議，更比往年增加了 3 個與臨床應用治癌新藥的 Clinical trial 之 Plenary section，其內容雖仍是新的癌症標靶治療藥物的相對初期成果，且參加此 Plenary section 的人員尚屬不多，但已算有別於過去 AACR 會議流於過度基礎研究，無法吸引臨床醫師的注意，常致研發結果有確實應用的困難。然而癌症研究的目的在瞭解癌症病情進展的精確機轉，以針對其機轉使用對的標靶治療藥物，因此若這類的研究成果無法讓治療癌症的醫師知悉且認同，將大大浪費了突破治療瓶頸的契機。因此大會規劃出與臨床結合的 Clinical section，自有其用心良苦之處。

本人於本次會議中，發表小鼠肺癌 Lewis lung carcinoma 的放射線引發轉移之 MMP-9 介入機轉，係引用 2001 年 Harvard group 發表的小鼠肺癌植入腿部，經放射治療腿部原發腫瘤獲得控制後，意外出現肺轉移情形。當初的研究認為此現象是原發腫瘤產生抑制全身轉移的 Angiostatin，在放射線控制原發腫瘤後不再產生，因而破壞了抑制血管新生的平衡，導致肺轉移的出現，也以 MMP-2 這個血管新生過程重要因子，來佐証其理論的成立。然而同樣的模型在我們的研究中發現，MMP-9 在放射線照射 Lewis lung
The expression of matrix metalloproteinases (MMPs) has been playing crucial roles in cancer progression and metastasis. This study is to clarify whether MMP associates with radiation-accelerated pulmonary metastasis of Lewis lung carcinoma (LLC-LM) in C57BL/6 mouse model. By using the in vitro matrigel-coated Boyden chamber invasion assay, non-cytotoxic dose of radiation (7.5 Gy) significantly enhanced LLC-LM cell invasiveness. Irradiated LLC-LM cells also showed the increased intravasation capability in the chicken chorioallantoic membrane assay. To test MMP family protein gene expression in irradiated LLC-LM cells, MMP-9 gene expression was significantly enhanced in both transcriptional and translational levels, so was the enzyme activity in
the culture supernatant. The use of anti-sense MMP-9 oligonucleotides significantly inhibited the *in vitro* radiation-enhanced invasiveness. With $1 \times 10^6$ MMP-9 RNAi stably transfected LLC-LM cells injected subcutaneously in the right thighs of C57BL/6 mice and irradiated to the primary tumor, the number of radiation-accelerated pulmonary metastases was significantly reduced (4.3 *versus* 23.0, p<0.001) (Table). The inhibitory effect of 1-benzyl-3-(5'-hydroxymethyl-2'-furyl) indazole (YC-1), through interrupting eIF-4E phosphorylation and MMP-9 expression, was shown on radiation-enhanced LLC cell invasiveness *in vitro*. YC-1 was further tested in the irradiated LLC-LM injected C57BL/6 mouse model for pulmonary metastasis. The number of radiation-accelerated pulmonary metastases was significantly reduced by pretreatment with YC-1 (daily dose of 10 mg/kg orally) starting 3 days before radiotherapy (1.6 *versus* 21.2, p<0.001) (Table). The serial urinary gelatin zymography demonstrated the therapeutic effect of YC-1 was likely to be through the inhibition of MMP-9 activity. We conclude that MMP-9 crucially involves the radiation-enhanced LLC-LM cell invasiveness *in vitro* and radiation-accelerated pulmonary metastasis *in vivo*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sham (wild-type LLC-LM)</th>
<th>Radiation to wild-type LLC-LM</th>
<th>MMP-9 RNAi transfected LLC-LM</th>
<th>Radiation to MMP-9 RNAi transfected LLC-LM</th>
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<tr>
<td>Number of pulmonary metastases</td>
<td>4.5±1.3</td>
<td>23.0±5.2</td>
<td>3.3±1.5</td>
<td>4.3±1.7</td>
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<tr>
<td>Treatment</td>
<td>Sham (wild-type LLC-LM)</td>
<td>Radiation to wild-type LLC-LM</td>
<td>YC-1 pre-treated LLC-LM</td>
<td>Radiation to YC-1 pre-treated LLC-LM</td>
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<td>Number of pulmonary metastases</td>
<td>5.4±1.8</td>
<td>21.2±6.6</td>
<td>1.6±1.1</td>
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