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学 位 論 文 題 名

The sustained elevation of Snail promotes glial-mesenchymal transition (GMT) after irradiation in malignant glioma

(Snailの持続的な上昇は悪性膠腫の放射線照射後のグリア間葉移行を促進する)

学位論文内容の要旨

[Background and Objectives] Glioblastoma (GBM) is the most frequent tumor of glial origin, but with a small general incidence and a dismally high mortality rate. Therapeutic modalities in general include surgical resection, fractionated radiotherapy as well as concomitant and adjuvant chemotherapy such as temozolomide. Even with aggressive treatment, the survival time is less than 15months with the survival rate at 3 years being less than 3%. Irradiation has a high cellular mortality and significant clinical improvement but specifically gliosarcoma (GSM) has been described as a subgroup of glioblastoma that has a biphasic phenotype and is frequent in recurring tumors. The epithelial-mesenchymal transition (EMT) phenomenon is a series of temporary and reversible events that occur in which there is loss of cell-cell adhesion, digestion of the extracellular matrix (ECM) and reorganization of the cytoskeleton with transcription and expression of mesenchymal genes. EMT has been well recognised for its role in phenotype switching as seen in developmental biology as well as its role in tumor progression and metastasis.

[Material and Methods] For immunohistochemical analysis, 22 paired patients samples were analysed and scored for glial markers (GFAP) and mesenchymal markers (Vimentin, α-SMA). Established commercially available cell lines were used for *in vitro* experiments where they were irradiated and the EMT transcription factors and mesenchymal markers were quantified by real-time PCR and Western blotting. The most prominent EMT related TF that was expressed in the short term and long term was then knocked down by siRNA. These altered cell lines were then irradiated again looking at the difference in the expression of mesenchymal molecules. Matrigel- and Transwell -invasion assay along with Wound healing assays were done to characterise the motility and invasiveness of these cells. Semi-quantitative PCR was done to measure the changes in stem cell markers due to irradiation with and without the siRNA. Immunofluorescense was used to visualise the cytoskeletal changes that occurred and to determine the change in stem cell marker. To correlate clinically with the *in vitro* findings real time PCR was done with 7 paired patients samples.

[Results] In the 22 paired patients samples, before irradiation, the tumors exhibited a glial morphology and immunohistochemistry, there was low expression of Vimentin and α-SMA and high amounts of GFAP, but these findings were reversed in the recurrent tumors after irradiation, the tumors now staining less for GFAP and more for Vimentin and α-SMA. The composition of the tumor had changed to consisting of elongated, bundled cells the looked more mesenchymal. After 10 Gy irradiation of *in vitro* samples, there was an increase in the EMT related transcription factors, Slug, Snail and Twist, after 48 hours, and 21 days after irradiation there was still a significant increase in the EMT factor Snail. There was also continuous cell death up to about day 12 after which the cells began to repopulate and proliferate. Knockdown of Snail

resulted in cells that expressed less mesenchymal molecules Col1A1, Col1A3 and α-SMA and less of a mesenchymal phenotype 21 days after irradiation and were not as invasive or mobile, this was also seen in the gelatin zymography, where there was an increase in MMP2 activity after irradiation in the cells that were not treated with siRNA. The activation of the mitogen-activated protein kinase (MAPK) pathway was demonstrated by the phosphorylation of ERK1/2 and the deactivation of the GSK-3β system was shown by its phosphorylation. Both of these effects were decreased with the use of siRNA to Snail. In these cells, there was an increase in the stem cell markers SOX2, NANOG and OCT3/4 after irradiation and this was abrogated with the use of siSnail. Immunofluorescence revealed that after irradiation there in increased decoupling of the Paxillin and Actin fibres, and again, this effect was abrogated by siSnail. Immunofluorescence also demonstrated an increase in the amount and intensity of cells that stained for SOX2. Analysis of 7 paired patients samples demonstrated the same pattern, a significant increase in Snail and mesenchymal molecules after irradiation.

[Discussion] Post irradiation recurrent tumors usually occur within the vicinity of the point of irradiation, as such, we decided to study cells that survive irradiation as these more reflect the clinical recurrent tumor. By immunohistochemistry we found that there is more mesenchymal features after irradiation, *in vitro* we were able to show that in the short term after irradiation (48 hours) there is increase in all of the EMT transcription factors that we examined (Slug, Snail and Twist) and 21 days after, there was a significant increase in Snail, that was still increasing after irradiation. Knockdown of this molecule 48 hours prior so that both the mRNA and protein levels were decreased at the time of irradiation abrogated any of the radiation mediated changes that were seen. An interesting effect was that even though we used siRNA once, the effect was noticeable after its lifespan, indicating that the initial increase in Snail is responsible for its long term increase and the changes that occur in relation. Mesenchymal molecules, stem cell markers and invasion all showed the same pattern that can be seen as Snail expression, after irradiation there was an increase in Snail, mesenchymal molecules, invasion, motility and stem cell markers, all these effects were decreased with the use of siSnail, even though this decrease was not uniform and identical, the pattern that arose where the weaker siRNA resulted in less of an abrogation than the stronger siRNA. Here we did not demonstrate an increase in TGFβ, as such we worked on the idea that this was a non-TGFβ phenomenon. Irradiation caused the activation of the MAPK pathway as shown by the increase of pERK1/2 and inactivation of GSK3β, shown by increase in p-GSK3β. This result is two fold, from the MAPK pathway there is an increase in the production of Snail and p-GSK3β results in less degradation, thus, resulting in an increase in the amount of Snail within the cell. The same phenomenon can be seen clinically where there is a significant increase in Snail and mesenchymal molecules after irradiation. This presents the situation where as a side effect to therapy, there is the creation of a more aggressive tumor and formation of stem cells. These stem cells, can differentiate into any cell type giving raise to a regrown tumor or a new tumor of different phenotype

[Conclusion] From our results it is clear that Snail is responsible for the mesenchymal changes, invasive phenotype and stem cell increase after irradiation especially in the latter phase and its knockdown abrogates these changes. As such, Snail might be a therapeutic target to inhibit mesenchymal changes after irradiation.

学位論文審査の要旨

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The sustained elevation of Snail promotes glial-mesenchymal transition (GMT) after irradiation in malignant glioma

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悪性神経膠腫は中枢神経原発悪性腫瘍のうち最も頻度が多く最も悪性度の高い腫瘍である。放射線治療は生命予後を改善するが、放射線治療後に残存した腫瘍細胞はその生物学的悪性度を増し治療抵抗性を示すことが報告されている。近年、悪性神経膠腫はその遺伝子プロファイルにより proneural, proliferative, mesenchymal の 3 タイプに分類され mesenchymal タイプに属する腫瘍は最も予後が悪く、再発時にはそのタイプに移行することが報告された。上皮性腫瘍では放射線照射後残存腫瘍では上皮間葉移行(Epithelial-Mesenchymal Transition:EMT)が起こりその運動能、浸潤能が増し転移能を獲得することが報告されている。転写因子である Snail, Slug, Twist は上皮の接着に関与する E カドヘリンの発現抑制、N カドヘリンの発現亢進などにより EMT を制御する。悪性神経膠腫は上皮性腫瘍ではないが、Snail, Slug, Twist などが発現しその悪性形質に関与していることが報告された。申請者らは悪性神経膠腫に対して放射線照射を行いこれらの転写因子の発現及び腫瘍の生物学的悪性度への関与を明らかにするために臨床病理学的・分子生物学的解析を行った。

WHO 分類により悪性グリオーマと診断された 22 名の患者の初発時の腫瘍及び再発時の腫瘍検体を用いてグリア細胞のマーカーである GFAP, 間葉系マーカーである Vimentin, α-Smooth muscle action (α-SMA)の免疫染色を行った。染色強度及び陽性細胞占拠率をスコア化し半定量的評価を行った。再発腫瘍では GFAP の発現減少、Vimentin 及びα-SMA の発現増加を認めた。次に悪性神経膠腫細胞株である T98G, KMG4 細胞を用いて *in vitro* の検討を行った。10Gy 照射後 48 時を初期変化、いったん細胞が減少し再増生が起きている照射後 21 日の細胞を晩期変化（再発）と想定した。それぞれの細胞から mRNA 及び蛋白を抽出し定量的 RT-PCR 法、Western blotting 法を用いて EMT 関連転写因子の発現解析を行った。いずれの細胞においても Snail は放射線照射 48 時間後、21 日後いずれの時

期でも上昇し、その上昇は蛋白量でも確認された。次に2種類のSnail SiRNAを用いて放射線照射後のSnailの発現への影響を同様な方法で調べた。Snail ノックダウン細胞は照射48時間後、21日後いずれにおいてもSnailの発現上昇を抑えた。また間葉系マーカーである1型コラーゲン、α-SMAの放射線照射48時間、21日後の発現上昇はSnailノックダウンによって抑制された。次に細胞骨格及び接着斑への影響を調べた。放射線照射後21日の細胞ではアクチンストレスファイバー、パキシリンの発現が増加したが、Snail ノックダウンによって減少が見られ運動能及び浸潤能も抑制された。また初発時のヒト悪性神経膠腫と比較して再発時の腫瘍では Snail、Type1 コラーゲン, ファイブロネクチン、MMP-2 の mRNA 発現が増加していた。次に放射線照射 21 日後の細胞における Sox2, Oct3/4, Nanog などの Stemness 遺伝子発現が上昇しておりSnailノックダウンによりこれらの発現が減少することが示された。これらの遺伝子発現の機序を調べたところ、放射線照射によってERK1/2及びGSK3βのリン酸化が亢進し、Snailノックダウンによってこれらの現象が抑制されることがわかった。過去の研究を合わせ、申請者らは残存腫瘍においては、放射線照射により誘導される活性酸素によりERKのリン酸化、GSK3βのリン酸化がおきSnailが高発現し、それがグリア間葉移行(Glial-Mesechymal Transition: GMT)、stemness 遺伝子が発現するというメカニズムを推測した。本研究によって、悪性神経膠腫への放射線照射の際にSnailの発現を抑制することが再発腫瘍の悪性化を制御できる可能性があることを示唆した。

審査員から神経膠芽腫における Vimentin の発現について、腫瘍周囲の正常細胞における変化について、患者検体におけるSnailの発現について、放射線照射線量を10Gyに設定した理由、T98G, KMG4細胞株の遺伝子プロファイル、Snailノックダウンが再発腫瘍の増加に与える影響、照射初期における stemness 遺伝子の発現などに関して質問があった。申請者はこれらの質問に対して自らの研究結果や先行研究の研究成果に基づいて概ね妥当な回答を行った。

審査員一同は、これらの成果を高く評価し、大学院課程における研鑽や取得単位なども併せ申請者が博士（医学）の学位を受けるのに充分な資格を有するものと判定した。