学位論文内容の要旨

博士の専攻分野の名称　　博士（医　学）　　氏名: Roshan Mahabir

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)

[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.