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Researchers are looking for a molecular profile that can predict *in vitro* responsiveness to EFGR inhibitors in patients with brain tumours

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From the bedside to the lab

THE NICB (National Institute for Cellular Biotechnology) – located on Dublin City University campus – has a long track record of cancer research and is one of the leading institutes in Ireland for translational cancer research.

While research has been focused mainly on non-neuronal cancers – for example, breast, lung and more recently pancreas and skin – tumours arising in the central nervous system, in particular in the brain, have not been studied at the NICB until recently.

Malignant brain tumours are one of the most virulent forms of cancer. They can result in serious physical and cognitive impairments. Brain tumours are often described as cancer of the soul, since the brain is the centre of thought, personality and emotion. Brain tumours are categorised into primary and secondary. Primary brain tumours originate in the brain and rarely spread outside the brain, whereas secondary tumours start outside the brain, for example in the lung, breast or skin, and metastasize to the brain.

Primary brain tumours

Primary brain tumours develop from brain cells and give rise to gliomas, meningiomas, central nervous system lymphomas, medulloblastomas, and tumours of the nerve sheet.

The majority, however, arise from glial cells, hence their name gliomas and account for 42% of all primary brain tumours and 71% of all malignant types.

Gliomas include oligodendrogliomas, ependymomas, and astrocytomas/glioblastomas, the latter representing the biggest group (about 80%). Astrocytomas are grouped according to their grade of malignancy:

- Pilocytic astrocytoma (grade I), which is mainly found in children
- Astrocytoma grade II, III
- Glioblastoma (grade IV) – which is most common in adults between the age of 30 and 70.

There are no socio-economic boundaries for the development of a these tumours.

Cause still unknown

The cause of primary brain tumours is still unknown. Exposure to ionising radiation and head injuries may be risk factors,

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as well as some inherited conditions for example neurofibromatosis, Von Hippel-Lindau syndrome, Li-Fraumeni syndrome, and Turcot's syndrome. However, only about 5% of all cases can be attributed to these causes.

In Ireland the incidence of primary brain tumours is 7/100,000 people per year (6.4/100.000 per year in the US).

There are about 316 new cases of primary brain tumours diagnosed every year in the Republic of Ireland and the mortality is around 67%. Despite major improvements in the surgical treatment the prognosis is poor.

Survival depends on the tumour grade and age. The five-year survival can be 65% for grade I and II and less than 10% for grade III and IV (glioblastoma) *(Cancer Atlas of the UK and Ireland 1991-2000)*.

The survival rate has improved very little in the recent decade. The median survival of glioblastoma (grade IV) is 189 days and has not significantly changed within a 12-year period (1993-2004).[1]

Treatment options for patients with malignant glioma were limited and used according to the principle 'one size fits all'; hence all subtypes were treated in the same way.

Treatment included a surgical attempt to remove visible tumour or at least reduce the tumour size (removal of as much visible tumour as possible) and radiotherapy, whereas chemotherapy consisting of a combination of procarbazine, vincristine, and CCNU (PCV) played a marginal role. In particular, glioblastoma (grade IV) was considered as highly chemoresistant.

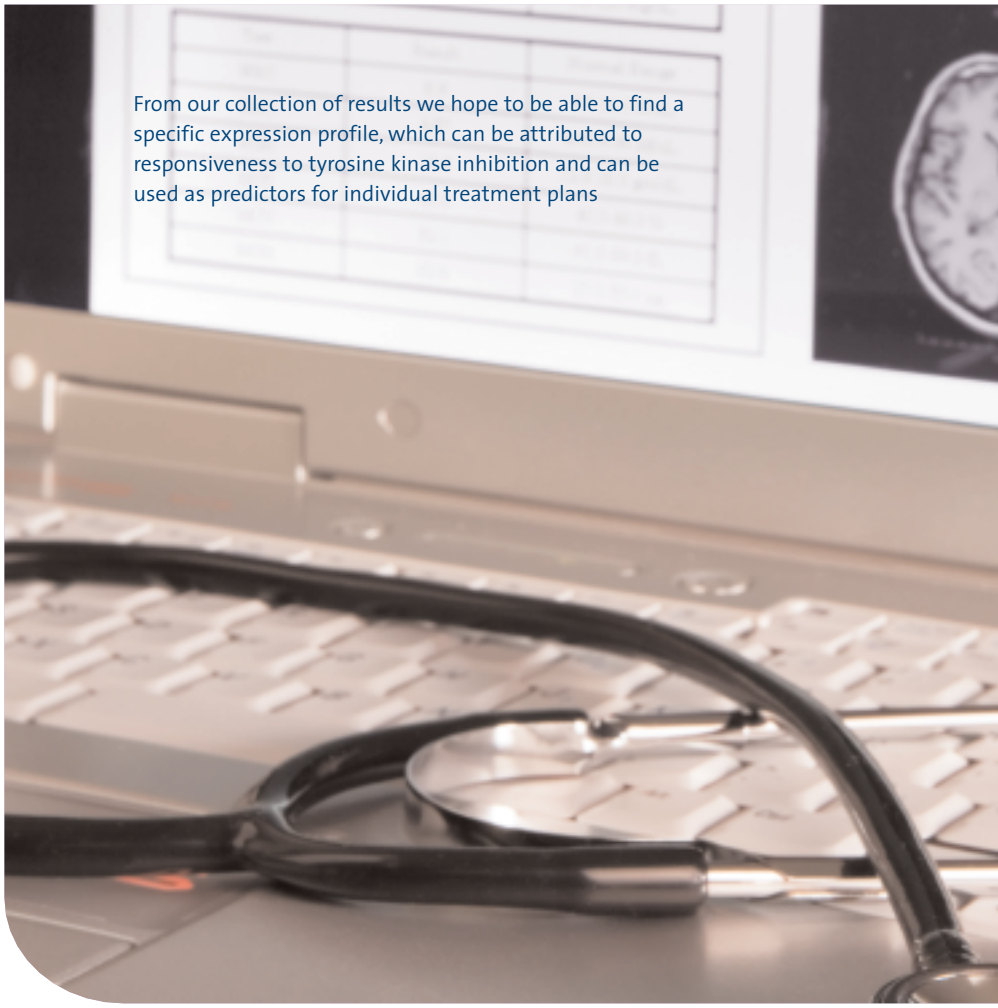
Due to the infiltrative nature of these lesions, recurrence is inevitable, even following an apparently complete surgical removal as checked by post-operative MRI or CT scan.

Sub-groups show more sensitivity

Many clinical trials using older or newer drugs show that there are often a small number of responders; whereas the majority of patients enrolled would not respond, which leads to the conclusion that the clinical trial failed.

In 1988, however, Cairncross and McDonald discovered that a subgroup of malignant gliomas revealed a better sensitivity to the combination chemotherapy PCV. Investigating the tumour tissue they found out that these tumours were characterised by deletions at chromosomes 1p and 19q[2,3] and that patients bearing these tumours would greatly benefit from the treatment with PCV.

Another more recent finding came from Stupp and his colleagues. They found that



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the methylguanine methyltransferase (MGMT) gene, which gives rise to a DNA repair enzyme, is correlated with the responsiveness to the newly developed drug temozolomide (Temodar, Temodal).[4,5]

Patients, who expressed the methylated form of the MGMT gene showed better overall survival than patients with the non-methylated form. The median survival was 22 months (versus 15 months) and the two-year survival rate was 46% (versus 23%).

Tyrosine kinase inhibitors

In 2005, Mellinghoff *et al* published the results of a clinical trial using the tyrosine kinase inhibitors erlotinib (Tarceva) and gefitinib (Iressa) in the treatment of malignant glioma. They reported 20% responders within the patient cohort, who showed a reduction in tumour size measured by MRI.[6]

Responsiveness to these tyrosine kinase inhibitors was associated with co-expression of EGFRvIII, a mutant variant of epidermal growth factor receptor (EGFR), and phosphatase and tensin homologue (PTEN), a tumour suppressor, which is often deleted in malignant gliomas. However, others have shown that responsiveness to EGFR blockade occurs independently of the presence of mutated EGFRvIII.[7]

The results of a small number of responders reflect the cellular and molecular heterogeneity of malignant gliomas. There is a need for further *in vitro* studies on patient tissue to be able to predict responsiveness to these types of targeted therapies.

Irish Cancer Society funding

In 2006, we were able to secure funding from the Irish Cancer Society for a three-year translational research project, which is performed in collaboration with the Neuropathology Department in Beaumont Hospital.

Together with consultant neuropathologist Michael Farrell we are trying to find a molecular profile, which is predictive for *in vitro* responsiveness to the EGFR inhibitors erlotinib (Tarceva), gefitinib (Iressa), and the platelet-derived growth factor receptor (PDGFR) inhibitor imatinib (Gleevec).

EGFR and PDGFR and their downstream effectors, for example Ras, Akt, and mTOR (mammalian target of rapamycin) are critically involved in the regulation of glioma survival, proliferation, invasion, and angiogenesis.

EGFR and PDGFR are attractive targets,

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because they are frequently amplified and/or over-expressed in malignant gliomas. Amplification of EGFR is often associated with the expression of a mutated variant EGFRvIII, which is constitutively active and growth factor independent. The expression of EGFRvIII is linked to increased expression of metalloproteinases and extra cellular matrix molecules, which results in increased invasiveness of the tumour.

Recent clinical trials with erlotinib and gefitinib have shown that there is a large variability in patient response. It became obvious that there was a subset of patients who responded much better to these inhibitors, however, the cause for this variability is not yet fully understood.

As yet there is very little data available on the in-vitro effects of EGFR and PDGFR/c-kit blockade on proliferation, invasion and expression of proangiogenic factors in glioma cells.

In vitro responsiveness

With our project we go from bed-side (patient) back to the lab by investigating patient tissue prior to any treatment except resection. We determine *in vitro* responsiveness to tyrosine kinase inhibitors by measuring their effects on proliferation, invasion and angiogenesis signalling, which represent the three cardinal biological characteristics of malignant gliomas. Ethical approval for this study was gained from Beaumont Hospital Ethics Board in 2005.

Candidate patients are identified on the basis of clinical presentation and imaging. With the help of a patient information leaflet the project is explained to these patients by a senior house officer of the Neuropathology Department and interested patients sign a written consent form prior to surgery. Patients, who have received tumour treatment before, are not considered for this study, except cases where tissue from the first intervention was already included in the study.

During operation, fresh tumour tissue is sent to the Neuropathology Department for intra-operative diagnosis. Having ensured that sufficient tissue has been harvested to allow for a definitive diagnosis, residual tumour will be split with 50% of the tissue snap frozen in liquid nitrogen and the other half placed into cold salt solution and transferred to the NICB laboratory.

Beaumont Hospital and NICB

This project is a two arm study. At Beaumont Hospital, Rachel Howley, a PhD student from the Royal College of Surgeons, performs immunohistochemical and molecular analyses on formalin fixed and on fresh frozen tissue, respectively. Antibody staining provides information about the expression status of EGFR, EGFRvIII, PDGFRα and β, PTEN, and a number of down-stream molecules within the signalling pathways.

Molecular analysis of genomic tissue DNA includes DNA sequencing, FISH (fluorescence *in situ* hybridisation), and real time PCR (polymerase chain reaction) producing information about gene expression levels of EGFR, EGFRvIII, PDGFR, and PTEN.

At the NICB, Paula Kinsella, a PhD student at the NICB, and I develop primary cell lines from the tissue samples. These cell lines are tested for their proliferative and invasive activity as well as for their sensitivity to the tyrosine kinase inhibitors erlotinib, gefitinib, and imatinib and to temozolomide (the chemotherapy drug currently used in the treatment of gliomas) and docetacel (Taxotere), a powerful chemotherapy drug used for a variety of other cancer types.

Using Western Blotting technique we determine protein expression of EGFR, EGFRvIII, PDGFR, and the same down-stream molecules, which are analysed in the tissue samples in Beaumont.

DCU grant

With an additional grant, secured in early 2007 from DCU's Faculty of Science and Health, we will analyse expression of microRNAs, which are small regulatory RNA fragments, in two groups of our primary tumour cell lines chosen according to their proliferative activity.

MicroRNAs have been identified to play a critical role in developmental and physiological processes and are implicated in the pathogenesis of cancer.

They represent small non-coding RNA molecules which can either act as oncogenes or as suppressor genes. In particular, miRNA-21 has been found to be involved in inhibiting apoptosis through PI3-kinase activation, which is dependent on the deletion of phosphatase and tensin homolog (PTEN) from chromosome 10.

Using low density arrays we will determine differences in microRNA expression between fast and low proliferating cell lines and functionally validate identified candidates in each cell line. This analysis adds another dimension to our translational study.

Results from both arms and the microRNA study are collected in a database and are compared to maximise the validity of *in vitro* glioma response data. The database also includes key clinical, radiologic, pathologic and – if available- follow-up information of each patient, which can be included into the analysis of the data.

Specific expression profile

From this collection of results we hope to be able to find a specific expression profile, which can be attributed to responsiveness to tyrosine kinase inhibition and can be used as predictors for individual treatment plans.

A secondary but important long-term goal is the development of reverse phase microarrays, which represents a rapid, high throughput technique for the characterisation of signalling pathways in human gliomas, which would allow a more accurate diagnosis and a personalised treatment plan.

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