Tumour hypoxia

Valorisation

Despite great advanced in cancer research, cancer is one of the leading causes of death in western society. In solid tumours, areas deprived of oxygen (hypoxia) arise due to an aberrant and immature vasculature. Tumour hypoxia is a common characteristic of solid tumours and leads to expansion of tumour cells with a more invasive, metastatic and therapy resistant phenotype, and is therefore a major clinical challenge. The work in this thesis describes mechanisms that hypoxic cancer cells use to sustain their survival and thereby contribute to progression of the disease. Understanding of these mechanisms is important so that hypoxia can be detected and therapies can be directed to target these more resistant cells and improve treatment outcome. As a result of innovative research in the industry and academia, cancer survival rates have increased dramatically over the last decades and have a positive societal impact.

Clinical relevance

Tumour hypoxia is clinically important as hypoxic tumours show resistance to most cancer therapies and are therefore associated with decreased patient survival. Typically hypoxia reduces the effectiveness of radiotherapy. Depressed oxygen levels in tissue lead to a decrease of long-lived reactive oxygen radicals after X- or γ-irradiation that are required for fixed DNA damage of the tumour cell. Consequently, hypoxic cells are three times more resistant to irradiation than well oxygenized cells. Additionally, hypoxic cells are resistant to chemotherapeutic drugs. Typically, chemotherapeutic drugs target rapidly dividing cells. Hypoxia reduces cell proliferation and thereby limits the effects of chemotherapeutic agents. Also the decreased drug delivery due to the aberrant vasculature in the tumour contributes to resistance of chemotherapeutics due to delivery limitations. Also the propagation of metastasis and reduced the effectiveness of surgery are linked to hypoxia. Tumour metastasis is associated with high patient mortality and is responsible for more than 90% of cancer-related deaths.

Taken together, hypoxia is one of the strongest contributors to patient's poor treatment outcome. Therefore it is highly desired to develop methods to determine hypoxia in a tumour. Such a method can be used as a marker for treatment response but is also required to stratify patients for the right therapy plan and for the development of therapies targeting hypoxia.

Hypoxia cannot be determined anatomically, as hypoxia is independent of tumour size, grade and stage. Currently, there are no markers for tumour hypoxia used in the clinic for diagnostic or treatment purposes. Invasive polarographic electrodes are the 'gold standard' for assessing tumour hypoxia. However due to the heterogeneous and dynamic properties of hypoxia, multiple samples must been taken in the diseased tissue, and are due to the high burden for the patient never used. Determining hypoxia by imaging markers, such as HX4 scanning or others have not shown adequate specificity or do not correlate with hypoxia at all. Other described methods are the use of deter-

mining osteopontin in patient fluids. However due the low elevation of this marker in hypoxic tumours compared to healthy subjects, this method is of limited use when applied to a single patient. Taken together, methods to determine hypoxia in a tumour are limited and are not implemented in current treatment plans, but also to stratify patients for hypoxia-targeted therapies hypoxia markers are highly desired.

Improvement for healthcare

The work described in this thesis can be exploited to modify tumour hypoxia to improve therapy outcome, but also to develop targeted therapies and prognostic biomarkers.

In chapter 5, findings are described that ATG12 could serve as a predictor for tumour hypoxia and a tumour control in head and neck cancer patients. We found that in 25-40% of patients with head and neck cancer, tumours lack ATG12 expression. The loss of ATG12 expression correlates with a decreased hypoxic fraction in the tumour. Strikingly, patients with ATG12 'negative' tumours show improved local and loco-regional control. Therefore, ATG12 expression can be used as a hypoxia predictor and be implemented in pathohistological analysis after biopsies. Such a marker can contribute to stratify patients for clinical trials investigating hypoxia targeted therapies. I.e. ATG12 negative tumours are expected to be less sensitive to these therapies and should therefore be excluded from these trials. Because ATG12 expression is lost in a substantial group of head and neck cancer patients, institutes investigating the effectiveness of hypoxia targeting therapies in clinical trials should be aware of this phenomenon before including patients in their trials. To investigate the effectiveness of hypoxia targeted therapies, stratification of patients with hypoxic tumours is required. The finding that ATG12 negative tumours have almost no hypoxic fraction could contribute to improved outcome of these trials.

In chapter 7 we describe our findings that hypoxic cells secrete a subset of extracellular vesicles that is marked by GABARAPL1 expression on their surface. This finding provides means for both diagnostic and therapeutic applications and marketable products: Since GABARAPL1^{+}EVs are released by hypoxic cells and are detectable in circulation of patients, GABARAPL1^{+}EVs can be used as a hypoxia marker. We already showed that GABARAPL1^{+}EVs are detectable and elevated in the blood of a small group (n=10) of colorectal, glioblastoma and small cell lung cancer patients. Currently our group is investigating if the number of circulating GABARAPL1^{+}EVs correlate with the degree of hypoxia in a tumour to evaluate if we can determine the amount hypoxia. Enzyme-linked immunosorbent assays (ELISA) are suitable assays to determine macromolecular species in fluids and are frequently used as a diagnostic tool. Currently our group is developing ELISA assays to detect GABARAPL1^{+}EVs in the blood of cancer patients. Such

a GABARAPL1^{+}EV ELISA can be translated in a marketable product and could be exploited as such. A GABARAPL1^{+}EV ELISA could provide information about the degree of hypoxia in a 'bench test' which is more desirable than invasive detection or expensive scanning methods which also require specialized personnel. Currently hypoxia activated pro-drugs (HAPs) are in development to specifically target hypoxic cells in a tumour. Tools to determine if a tumour is hypoxic are highly desired for patient stratification for these clinical trials.

The finding that GABARAPL1 is expressed on EVs also has therapeutic applications. Since GABARAPL1 is expressed on the surface of hypoxic EVs, it is accessible for targeting, for instance with antibodies. We already showed in chapter 8 that inhibition of GABARAPL1 with hairpin RNA's decreased metastasis formation by 80% in a metastatic breast cancer model in mice. Additionally, targeting GABARAPL1 resulted in decreased angiogenesis, tumour growth and tumour regrowth after irradiation and importantly, GABARAPL1^{+}EVs are detectable and elevated in the circulation of cancer patients compared to healthy controls (chapter 7).

Therefore, we hypothesize that targeting GABARAPL1^{+}EVs in the patient's circulation by GABARAPL1 targeting antibodies can reduce the formation of metastasis, but also might affect growth of the primary tumour; two important goals to achieve in patients. In chapter 7 we showed in a proof of concept experiment that we are able to block GABARAPL1^{+}EV with antibodies targeting GABARAPL1 *in vitro* and thereby inhibiting their pro-angiogenic properties. Extrapolated to patients, this could include that this approach might prevent the formation of a pre-metastatic niche, and thereby *prevent* the development of metastasis.

These *in vitro* experiments were done with commercially available polyclonal antibodies. For therapeutic applications, typically monoclonal antibodies are used. Most used antibody subtypes are IgG1, 2 or 4, due to their long half-life in circulation (up to 21 days). We already have developed GABARAPL1 targeting monoclonal antibodies to further explore and validate this approach in *in vitro* and *in vivo* studies.

Since the use of the first monoclonal antibody in 1986, the market for these biopharmaceutical drugs has grown significantly. At present, 47 antibodies have been approved in the US or Europe. Annually, 4 new antibodies achieve marked approval, leading to an estimated world-wide sale of nearly $125 billion in 2020 [1]. However, currently there are no therapeutics on the marked or clinical trials investigating drugs targeting endogenous extracellular vesicles. In that perspective, this method could lead to the development of a novel and innovative anti-cancer therapy. The method to target EVs, and more specifically GABARAPL1^{+}EVs, to prevent the formation of metastasis is described in a patent in Europe and the US (Keulers & Rouschop, patent number WO2015121295A1). To further develop this approach and translate this into a possible treatment we are looking for research partners to support further (co-)-development and clinical proof of concept treatment. Such collaboration could lead to a university spin-off company to explore its clinical feasibility and efficacy.

GABARAPL1^{+}EV targeting antibodies could be applied as a neoadjuvant therapy on patients with solid, and thus hypoxic, tumours. It needs to be empirically determined if GABARAPL1^{+}EVs indeed are secreted by most solid tumours, but it is expected that most tumours release GABARAPL1^{+}EVs. Most cancer related deaths are attributed the metastatic disease. Typically, distant metastases are frequently unresponsive to existing therapies and are treated systemically with palliative intent. Preventing the formation of metastasis could improve the life expectancy of many patients.

Tumour hypoxia contributes to poor treatment outcome in several cancer types. Therefore, reducing the hypoxic fraction in a tumour is highly desired. Attempts to modulate the hypoxic fraction were primarily focussed on increasing oxygen supply to the tumour. A different approach is alleviating hypoxia by targeting cellular mechanisms that use cells for their survival, such as autophagy.

Chloroquine is originally marketed as an anti-malaria drug, but is also used in the treatment of rheumatoid arthritis and lupus-associated arthritis. In addition to these properties, chloroquine is a potent autophagy inhibitor. Previously we showed that chloroquine sensitizes cells to hypoxia, reduces the hypoxic fraction and sensitizes xenograft tumours to radiotherapy in mice [2].

In chapter 4 we show that EGFRvIII expression elevates the activation of autophagy during hypoxia. The mutant form of EGFR, EGFRvIII is frequently observed in glioblastoma (50-60% of patients with EGFR amplification) and is associated with increased therapy resistance and poor prognosis. A retrospective study indicated that patients with EGFRvIII positive tumours benefit most of concurrent chloroquine treatment (median survival form 3 to 15 months).

Currently, a phase I clinical trial is ongoing in our institute (clinical trial NCT02378532) to determine the maximum tolerated dose of chloroquine with concurrent radiotherapy and temozolomid. So far, this trial shows promising results. To determine if chloroquine can be repurposed as a drug in oncology and considered as an additional treatment for GBM patients, additional clinical trials are required to investigate efficacy, effectiveness and safety.

References

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2. Rouschop, K.M., et al., *The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5.* The Journal of clinical investigation, 2010. 120(1): p. 127-41.