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NRF2 and oxidative stress in melanoma – implementation of an analytic platform

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Abstract

Our group investigates the role of NRF2, a key mediator of the oxidative stress response, in melanoma. The system we are presenting is based on modulation of the transcription factor NRF2 and its primary regulator protein KEAP1 by transient siRNA transfection. Therefore it allows us to activate or deactivate the oxidative stress response of the cell. It is going to serve as important part of the toolbox we will use to further investigate the influence of oxidative stress in melanoma in terms of migration potential and treatment resistance.

Keywords:

Melanom, Oxidative Stress, ROS, NRF2, KEAP1

Introduction

Though the last years have seen promising developments especially on the field of immune therapy, malignant melanoma remains a big challenge for medicine. Despite accounting for just 1% of skin cancers diagnosed, melanoma is responsible for most skin cancer related deaths. According to the World Health Organization there are 132000 newly diagnosed cases of melanoma per year and approx. 50 percent of patients do not respond to current therapy at advanced stages. Melanoma is still one of the most deadly cancer types with approximately 10 percent mortality five years after diagnosis.

The high mutation burden plays a crucial role in terms of survival. A prominent example is the oncogenic mutation of B-RAF and its downstream signalling pathway, which is key prerequisite for inhibitor treatment like Vemurafenib (Ravnan & Matalka 2012). Besides the further implementation of patient specific therapies, which take these mutation states into account, the development of therapies targeting broadly applicable mechanisms need to be developed. A large percentage of patients would benefit, as such mechanisms can be included in a larger portion of personalized therapy approaches. During this process basic cellular characteristics are identified as possible targets. Elevated oxidative stress is such a process, which is shared by a large percentage of tumor types. This elevated stress levels might be due to

enhanced metabolic activity or hostile microenvironments within the tumor. However, regular or tumor cells have the ability to protect themselves against oxidative stress caused by Reactive Oxygen Species (ROS) and thereof resulting cellular damage. While this mechanism is a welcome protection and tumor suppressor in regular cells, tumor cells use it to enhance their resistance against various treatments and even boost their proliferation and metastatic properties (Sosa et.al 2013).

A key mediator in this protective response system is the transcription factor NRF2, also termed Nuclear-factor-erythroid-2-related-factor-2. NRF2 is a transcription factor of the Cap 'N' Collar (CNC) family and has a basic leucine zipper (bZIP) structure. It recognizes the Antioxidant Response Element (ARE) as Promoter. This promoter is in charge of a broad variety of functional mechanisms like for example the glutathione system and the NADPH Synthesis which are strongly involved in cellular redox homeostasis. Beside its role in ROS management, NRF2 also triggers detoxification and excretion mechanisms, which are especially problematic in tumor cells as they contribute to resistance formation. Also metabolism is altered in a way that cell growth and proliferation is favoured. The pentose phosphate pathway (PPP), for example, is strongly activated in order to generate building blocks for cell growth and the creation of glutathione for ROS elimination also requires additional output from the citric acid cycle (Mitsuishi et.al. 2012).

Many of these mechanisms are permanently active in tumor cells, which makes NRF2 and oxidative stress a valid target for closer investigation. Also various therapeutics used in cancer treatment elevate the levels of oxidative stress within cells. Therefore treatment of tumor cells able to excessively activate the NRF2 response to counteract this treatments are a great challenge. Indeed alterations of this pathways activity have been found in tumors of various types which were shown to contribute to the tumors survival potential (Menegon et. al. 2016). The alterations found so far include its primary regulator protein KEAP1 as well as accumulation of disruptor proteins and onco-metabolites. The majority of mutations found was located in the domains responsible for the interaction between NRF2 and KEAP1.

This interaction between NRF2 and KEAP1, short for Kelch-like-ECH-associated-protein-1, is the central mechanism which regulates the response of the pathway and induces the effects described before. NRF2 is constantly expressed and located in the cytoplasm where it is bound to KEAP1 which in turn serves as mediator for the Ubiquitin ligase Cullin3. Via this setup NRF2 is tagged with several Ubiquitin residues which mark it for degradation in the proteasome. This prevents it from reaching the nucleus and from performing its role as transcription factor. If a stress signal is present KEAP1 is modified to no longer mediate the ubiquitination, according to the two current models either by partly detaching from NRF2 or by not recruiting cull3 anymore. To achieve this effect KEAP1 is directly modified by electrophiles at its various cysteine residues (Kansanen et.al. 2013). This regulatory mechanism is quite similar to those of NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) and shares the advantage of allowing for a fast response via imminent stabilization of the otherwise degraded effector molecule upon spontaneous occurrence of dangerous conditions, in this case elevated oxidative stress. Further regulatory mechanisms include primarily phosphorylation of NRF2 and KEAP1 which are capable of influencing activity as well as degradation.

Methods

For our studies we used isogenic, human patient-derived melanoma cell lines. (Swoboda et.al; Schütz et.al. 2016) Protein expression was measured by western blotting and protein localization was illustrated by confocal immunofluorescence. Target gene expression was measured by Taq-man real time PCR.

We aimed to develop a platform where NRF2 can be switched on and off. Therefore our strategy was to targeted NRF2 or KEAP1 with siRNA oligos (five different oligos were mixed to target a single gene).

In case NRF2 is targeted this lead to reduced protein levels within the cells. By transiently transfecting melanoma cell lines with siRNA directed against KEAP1, we generated cells which constantly activate the NRF2 pathway. A further option would have been a stable integration, but since NRF2 is a major player in the cell, which effects a broad variety of targets, a chronic change would strongly alter the properties of the cell line in general. A transient transfection works instantly and still allows for a generous timeframe to conduct experiments.

In order to properly assess the activation of the NRF2 pathway via stabilization of the transcription factor we tested ROS inducer compounds. Among these Pyocyanin, a known inducer of oxidative stress related responses, resulted in the strongest activation, visible in an increase of NRF2 signal intensity on western blot. The reduction of KEAP1 protein levels by siRNA treatment resulted in a comparable increase in NRF2 levels.

By specifically targeting the two interaction partners NRF2 and KEAP1 we have generated a platform where we either activate the oxidative stress response by default or prevent it from being activated at all. In this setting drugs and substances which would usually cause a strong response of the NRF2 pathway can be tested without doing so and others which would not do so can be tested with the response active. Furthermore we intent to perform a screening approach and to evaluate if synergistic effects can be achieved by combining specific treatment-compounds with up- or down-regulation of the oxidative stress response via NRF2. Readouts of this tests will mainly be growth and proliferation data as well as cytotoxicity and cellular apoptosis.

Results and Discussion

Here we showed that ROS inducers lead to an activation of the NRF2 pathway in melanoma cells. Furthermore, we were able to block NRF2 target gene expression by siRNA mediated silencing of NRF2. By targeting of KEAP1 we increased NRF2 protein levels and up-regulated NRF2 target genes. Therefore depletion of NRF2 abolishes the ability of the cell for antioxidant defence and paves the way to perform screening of therapeutically relevant compounds for synergistic effects on cell survival. As stated previously several cancer types have high levels of NRF2 activation. Especially metastatic tumors might depend on a functional and highly active protective system against oxidative stress since metastasising cells are exposed to an extremely hostile environment during the process. (Piskounova et.al. 2015) By analysing changes in mRNA levels of downstream target genes of the NRF2 pathway and possibly by applying proteomic approaches the networks which mediates the oxidative stress during metastasis can be further investigated. Of course the metastatic potential itself can be evaluated in functional assays and changes in the migratory and invasive potential of the transfected cells can be characterized. Due to the inclusion of highly aggressive as well as non-aggressive tumor cell lines in the primary setup a wide range of possible shifts in metastatic potential is covered. This can be used to investigate the role of oxidative stress during the advancing development of tumor aggressiveness.

Finally pharmacological inhibitors of NRF2 can be tested against the siRNA treated cells and compared in their effectiveness. As possible treatments options for patients focusing on NRF2 would most likely rely on one of these inhibitors. Several inhibitors derived from natural and chemical sources can and need to be tested for this purpose.

In summary we established a platform to modulate NRF2 response in melanoma cells. This will enable us to identify potential novel therapeutic options especially for melanoma patients not responding to current state of the art treatments.

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