Minireview

Grainyhead-like transcription factors in cancer – Focus on recent developments

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Impact statement

In the present article, we focus on relatively little appreciated aspects of involvement of the grainyhead-like (GRHL) transcription factors in cancer. These aspects are nevertheless very important for the functioning of GRHL proteins, as well as for cancer development. Some of the GRHL factors perform tumor-promoting functions in certain types of cancer, which makes them potential drug targets. Much information is available about somatic cancer mutations in the *GRHL* genes, yet there are very few analyses of these mutations in the scientific literature. The activity of GRHL transcription factors is controlled by phosphorylation, and we suggest that regulating their phosphorylation with specific protein kinases provides an alternative approach to modify the activity of GRHL proteins. Some single nucleotide polymorphisms (SNPs) in the *GRHL* genes are associated with disease risk. Studying such SNPs may yield new information about the functioning of *GRHL* genes and proteins, and may also allow to identify people with an increased risk of a particular disease.

Abstract

The role of grainyhead-like transcription factors in cancer has been widely investigated by the scientific community. However, some of its aspects do not seem to be adequately appreciated, and these are the topic of our article. In addition to their well-documented role as tumor suppressors, in many cases the grainyhead-like proteins perform tumor-promoting functions, which make them potential drug targets. However, it is difficult to directly target transcription factors, which is why we recommend an alternative approach. The transcriptional transactivation activity of grainyhead-like transcription factors is regulated by phosphorylation, and protein kinases are much more feasible drug targets. Studying the phosphorylation of grainyhead-like proteins may thus allow to identify protein kinases regulating the activity of these factors, and design inhibitors of these kinases to indirectly regulate the activity of grainyhead-like transcription factors. There are many somatic mutations in the *GRHL* genes that occur during cancer development. These mutations are widely distributed across the *GRHL* loci, and these mutations are very rare. For this reason, they are unlikely to become targets of future therapies, nevertheless some of them may be driver mutations and studying them may provide important novel information about the regulation of functioning of the *GRHL* genes and proteins. Analogous information may be obtained by studying single nucleotide polymorphisms in *GRHL* genes that are associated with disease risk. Such polymorphisms may also prove useful in identifying individuals with an increased risk of a particular disease.

Keywords: Drug target, grainyhead-like, oncogene, transcription factor, tumor suppressor

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Introduction

The involvement of the grainyhead-like (GRHL) transcription factors in cancer is very well documented, and is a subject of almost 200 publications listed in the PubMed database. Many review articles have recently been published on this topic.[1–4] These articles discuss many of the important aspects of GRHL involvement in cancer. For this reason, we decided to focus our review on those GRHL characteristics that do not receive enough attention, yet in our opinion are very important. Many of these features have been studied in our laboratory.

Functions of grainyhead-like transcription factors in mammalian development

The grainyhead-like (GRHL) transcription factors constitute a family whose first member, Grainyhead (GRH), was discovered in the fruit fly *Drosophila melanogaster*.[5] We and our collaborators were the first to identify and

describe the mammalian orthologs of GRH.[6,7] These orthologs are found primarily in epithelial tissues, in organs such as epidermis, oral and olfactory epithelium, kidneys and urogenital tract, stomach and the digestive tract, heart, and lung.[8] We have subsequently generated mouse models to investigate the roles of GRHL transcription factors in mammals. These include knockout strains of *Grhl1*, *Grhl2* and *Grhl3*; conditional knockouts of *Grhl2* and *Grhl3*, and knockin of *Grhl2* into the *Grhl3* locus. The analysis of these models provided fundamental information about the functions of GRHL proteins in mammals. The GRHL1 transcription factor is important for the functioning of the epidermis, as the *Grhl1*$^{-/-}$ mice exhibit palmoplantar keratoderma, impaired hair anchoring and desmosomal abnormalities.[9] Loss of *Grhl2* causes early embryonic lethality at approximately day E11.5 with severe neural tube closure defects.[10] The GRHL3 factor is essential for neural tube closure, epidermal barrier formation, and cutaneous wound healing, and the *Grhl3*-null mice die at birth with spina bifida and severe skin barrier defects.[11–13] Combined deletion of *Grhl1* and *Grhl3* in adult mice leads to postnatal loss of impermeable skin barrier and death.[14]

Grainyhead-like factors acting as oncogenes

In most cases, the GRHL transcription factors act as tumor suppressors; many such examples were extensively discussed in recent reviews.[1,3] However, in some instances they display oncogenic functions; most known examples involve GRHL2. The expression patterns of GRHL2 in various tumors are very complex.[15] They may reflect potential tumor-suppressing as well as oncogenic functions of GRHL2 in distinct types of cancer. Historically, the first case in which GRHL2 may serve as an oncogene was reported for hepatocellular carcinoma (HCC). A copy number gain of *GRHL2* was associated with early recurrence of HCC. In addition, silencing the expression of *GRHL2* in HCC cells inhibited their growth, suggesting a link with cell proliferation.[16]

One of the best characterized examples of GRHL2 oncogenic functions concerns its involvement in oral squamous cell carcinoma (OSCC). GRHL2 regulates the expression of human telomerase reverse transcriptase (*hTERT*) gene in OSCC cells, suggesting a role in telomerase activation during cellular immortalization.[17] The levels of GRHL2 are increased in human OSCC tumor samples, and silencing the expression of *GRHL2* in OSCC cells results in the loss of *in vivo* tumorigenicity and cancer stemness.[18] Very interesting discoveries were made using a conditional *Grhl2* knockout mouse model, in which the *Grhl2* gene was specifically ablated in epithelial tissues.[19,20] Such mice do not display any gross phenotypic defects, but they are resistant to chemically induced oral tumorigenesis, in contrast with wild-type controls. These findings suggest that inhibiting GRHL2 activity in adult organisms is unlikely to cause any harm to healthy cells and tissues, but may be useful in the treatment of cancer. Subsequent analyses determined that GRHL2 activates the Erk and JNK MAP kinases, which in turn suppress the TGF-β signaling pathway.[19] Another pathway in these mice involves direct transcriptional regulation by GRHL2 of expression of forkhead box M1B (*FoxM1B*), a known oncogene essential for cell cycle progression and tumorigenesis.[20]

In the case of colorectal cancer (CRC), increased levels of GRHL2 were found in CRC samples obtained from human patients. The expression of GRHL2 correlated with higher levels of Ki-67 staining, larger tumor size, and advanced clinical stage.[21,22] Silencing of *GRHL2* expression in CRC cell lines decreased cell proliferation, induced apoptosis, and impaired tumorigenesis in a nude mouse xenograft model.[21,23] Consistently with the above, ectopic overexpression of *GRHL2* in CRC cell lines increased cellular proliferation *in vitro* and promoted tumor growth *in vivo*.[22] It was proposed that GRHL2 can be used as a novel predictive biomarker for both overall survival and recurrence-free survival, and represents a potential therapeutic target against CRC.[21,22] On the other hand, *GRHL2* knockdown in CRC cells promoted migration, invasion, and metastasis in a mouse model, which is consistent with a tumor suppressive role.[24] We suppose that the oncogenic function of GRHL2 in CRC probably depends on the stage of disease progression.

The *GRHL2* gene is hypomethylated in high-grade serous epithelial ovarian tumors, and it is strongly overexpressed in both low malignant potential and high-grade serous epithelial ovarian tumors.[25] Silencing of *GRHL2* expression decreases proliferation, migration, and invasion and induces G1 cell cycle arrest in epithelial ovarian cancer cells. The above findings indicate strong oncogenic potential of GRHL2 in epithelial ovarian cancer progression.[25] In non-small cell lung cancer (NSCLC), patients with high GRHL2 levels were associated with poor prognosis compared to patients with low GRHL2 levels. *GRHL2* overexpression promoted cell growth and colony formation, and simultaneously suppressed cell migration in NSCLC cells.[26] In prostate cancer, GRHL2 functions as an enhancer of the oncogenic androgen receptor signaling pathway, but it also suppresses metastasis-related phenotypes.[27] The levels of GRHL2 are increased in pancreatic cancer samples; they are correlated with metastasis and poor survival.[28,29] The levels of GRHL2 in esophageal cancer samples are higher than in adjacent tissues and are associated with tumor differentiation. They are significantly correlated with lymph node metastasis and invasion depth.[30] Finally, GRHL2 acts as an oncogene in some subtypes of breast cancer, which was extensively discussed in a recent review.[4]

GRHL3 is yet another member of the GRHL family that displays oncogenic functions in some cancer types. In colorectal cancer, increased expression of *GRHL3* was observed in both tumor samples and cell lines, and silencing of *GRHL3* suppresses CRC cell proliferation, viability, and migration *in vitro*, and can induce apoptosis in some CRC cell lines.[31] In diffuse large B-cell lymphoma, the five-year survival rate in patients with high GRHL3 levels was significantly lower than in those with non-detectable GRHL3 levels, and multivariate analysis identified GRHL3 expression as an independent predictor of poor survival.[32]

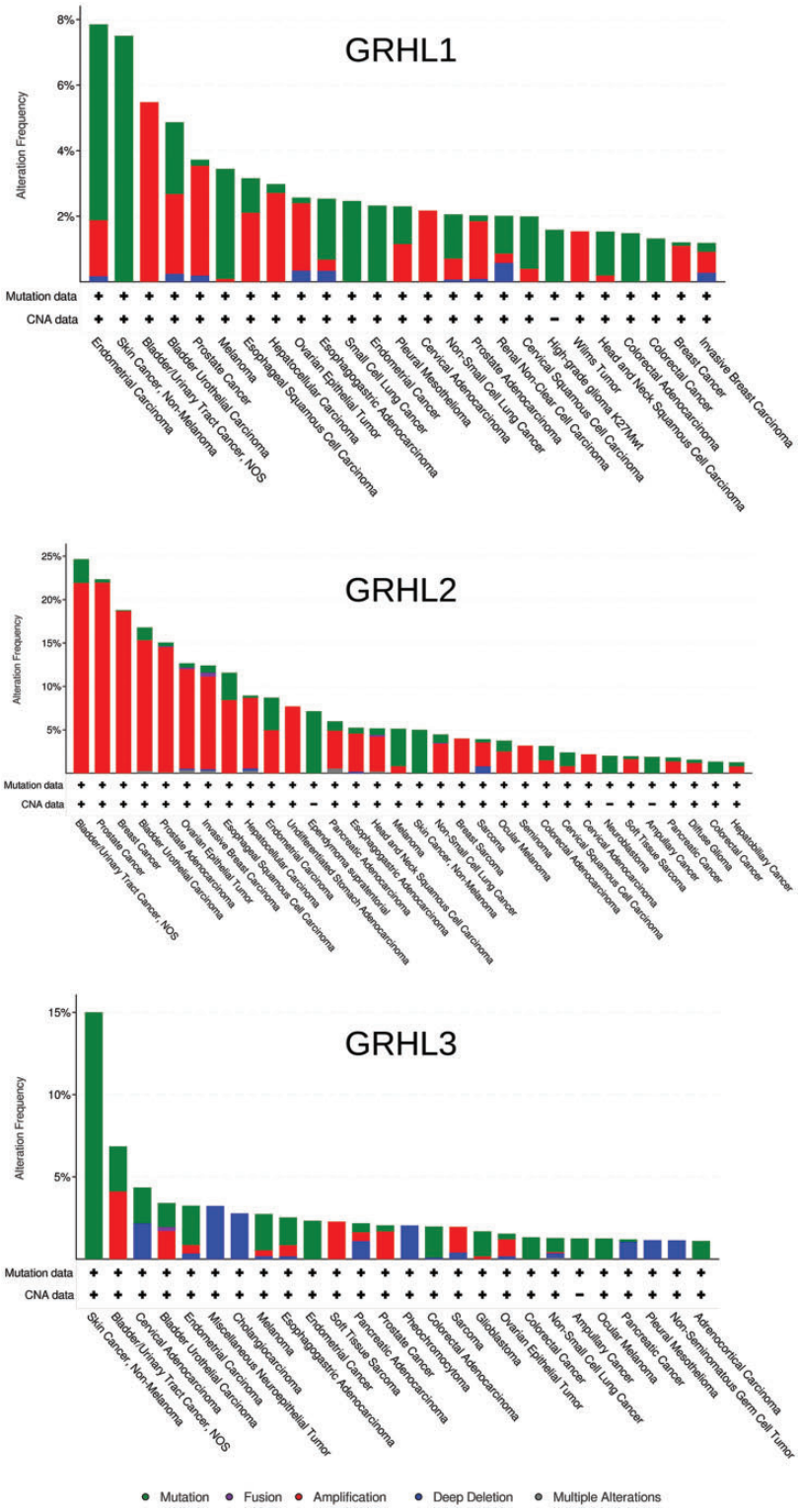

Figure 1. Percent of cancer cases with genomic alterations in *GRHL* genes. Only cancer types with a minimum of 1% altered cases are shown. The data were obtained from the curated set of non-redundant studies containing 42,027 samples, available at the cBioPortal for Cancer Genomics (www.cbioportal.org, accessed October 2019).[37,38] Mutations are indicated in green, fusions in purple, amplifications in red, deep deletions in blue, multiple alterations in grey.

Somatic mutations in grainyhead-like genes in cancer

In our earlier research, we did not find any somatic mutations in tumor samples obtained from patients with non-melanoma skin cancer (NMSC) and clear cell renal cell carcinoma (ccRCC).[33,34] The first such mutation was reported in early onset luminal breast cancer.[35] It was a nonsense mutation near the 5′ end of the coding region of the *GRHL2* gene, p.E32*. This mutation is expected to be strongly pathogenic, as it completely abolishes the

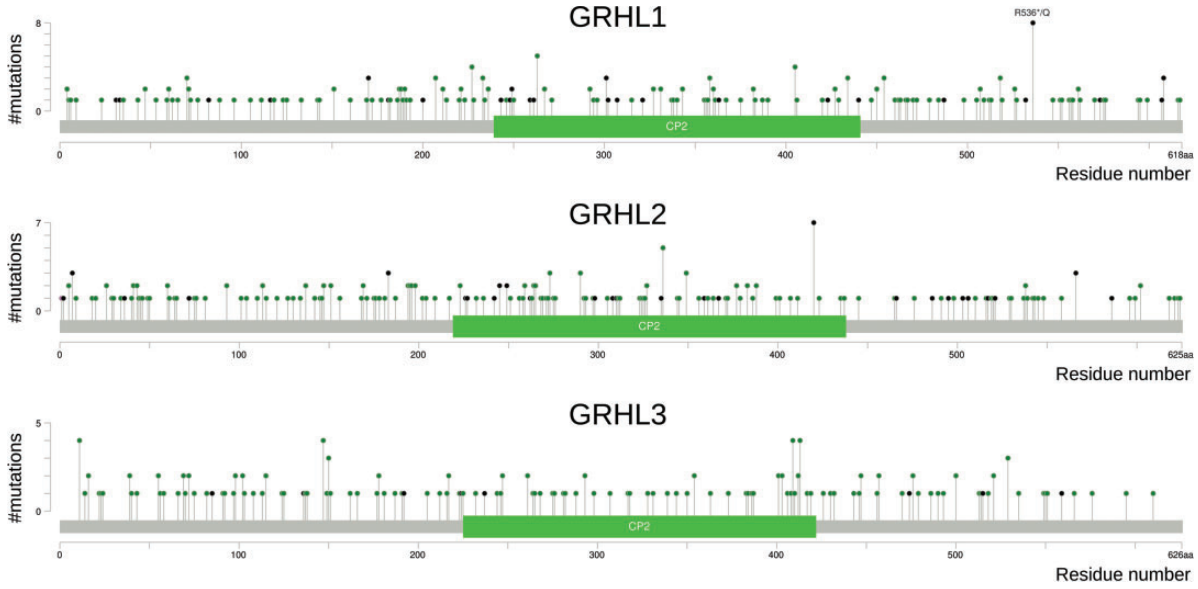

Figure 2. Distribution of somatic mutations in the *GRHL* genes in human cancer samples. The data were obtained as in Figure 1. Missense mutations are indicated in green, truncating mutations in black, other mutations in purple.

functionality of GRHL2 protein – a truncated protein would lack all the functional domains: transactivation domain, DNA-binding domain as well as dimerization domain. Furthermore, a transcript carrying such a mutation is expected to undergo nonsense-mediated decay.[36] It was proposed that this is possibly a driver mutation in this type of cancer.[35] However, this mutation occurs at a very low frequency of only 0.79% in breast cancer.[35]

Another such mutation is p.R427Q in GRHL1, which was found in stomach adenocarcinoma and lung adenocarcinoma samples (www.cbioportal.org, accessed October 2019).[37,38] It was shown that the p.R427 residue is crucial for the binding of the GRHL1 transcription factor to DNA, and the p.R427Q substitution strongly reduces the affinity of GRHL1 to its target DNA sequence.[39] It is thus likely that GRHL1 acts as a tumor suppressor in these cancer types and this substitution, by reducing its DNA-binding affinity, impairs its tumor suppressive function.

The cBioPortal for Cancer Genomics contains information about genomic alterations in human cancer samples (www.cbioportal.org, accessed October 2019).[37,38] We summarize the data regarding the *GRHL* genes in Figures 1 and 2. There are clear differences between various *GRHL* genes: *GRHL2* is frequently amplified in multiple cancer types, while such alterations occur more rarely in *GRHL1* and rarer still in *GRHL3* (Figure 1). However, such amplifications may involve many genes and therefore it is difficult to interpret the importance of individual loci within the amplified regions for the cancer development.

Overall somatic mutation frequency in cancer in these loci is low: 0.5% for *GRHL2*, 0.4% for each *GRHL1* and *GRHL3*. However, this strongly depends on the cancer type, as the *GRHL* genes are the most frequently mutated in NMSC: 15% (6 out of 40) cases for *GRHL3*, 7.5% (3 out of 40) cases for *GRHL1* and 5% (2 out of 40) cases for *GRHL2*, which is consistent with our earlier findings indicating a crucial role for these genes in NMSC.[33] The *GRHL* genes are also frequently mutated in another type of skin cancer, malignant melanoma: *GRHL2* in 4.3% (49 out of 1132) cases, *GRHL1* in 3.4% (38 out of 1132) cases and *GRHL3* in 2.2% (25 out of 1132) cases. The data from the cBioPortal for Cancer Genomics also indicate potential involvement of *GRHL* genes in some types of cancer to which these genes have not been linked before. The *GRHL* genes are frequently mutated in endometrial carcinoma: *GRHL1* in 6.0% (35 out of 586) cases, *GRHL2* in 3.8% (22 out of 586) cases and *GRHL3* in 2.4% (14 out of 586) cases. Another interesting finding concerns bladder urothelial carcinoma, with 2.2% (9 out of 411) mutated cases for *GRHL3* and 1.5% (6 out of 411) cases for each *GRHL1* and *GRHL2* (Figure 1). In summary, somatic mutations in the *GRHL* genes in cancer are common overall, therefore for this as well as other reasons presented in this article, these genes fit the classic definitions of oncogenes or tumor suppressors.

Interestingly, although the *GRHL1* gene is frequently mutated in various cancers (Figures 1 and 2), it was never reported to act as an oncogene, but in many cancers it acts as a tumor suppressor.[33,34,40,41] We can therefore assume that those mutations in the *GRHL1* gene that are relevant to cancer development are loss-of-function mutations which impair its tumor suppressive functions.

Even though the *GRHL* genes are frequently mutated in some types of cancer, there are no cancer mutation hotspots in these genes (Figure 2). Instead, numerous mutations are evenly spread across loci, and these mutations are very rare. The most common is nonsense mutation pR536* in *GRHL1*, which was found in only about 0.01% of all cancer samples included in the curated set of non-redundant studies at the cBioPortal for Cancer Genomics (in the total of six samples). Therefore, it is unlikely that therapies targeting individual mutations in the *GRHL* genes will be developed in the foreseeable future, as these mutations occur extremely rarely. However, it may be feasible to design small molecule inhibitors of GRHL2 and

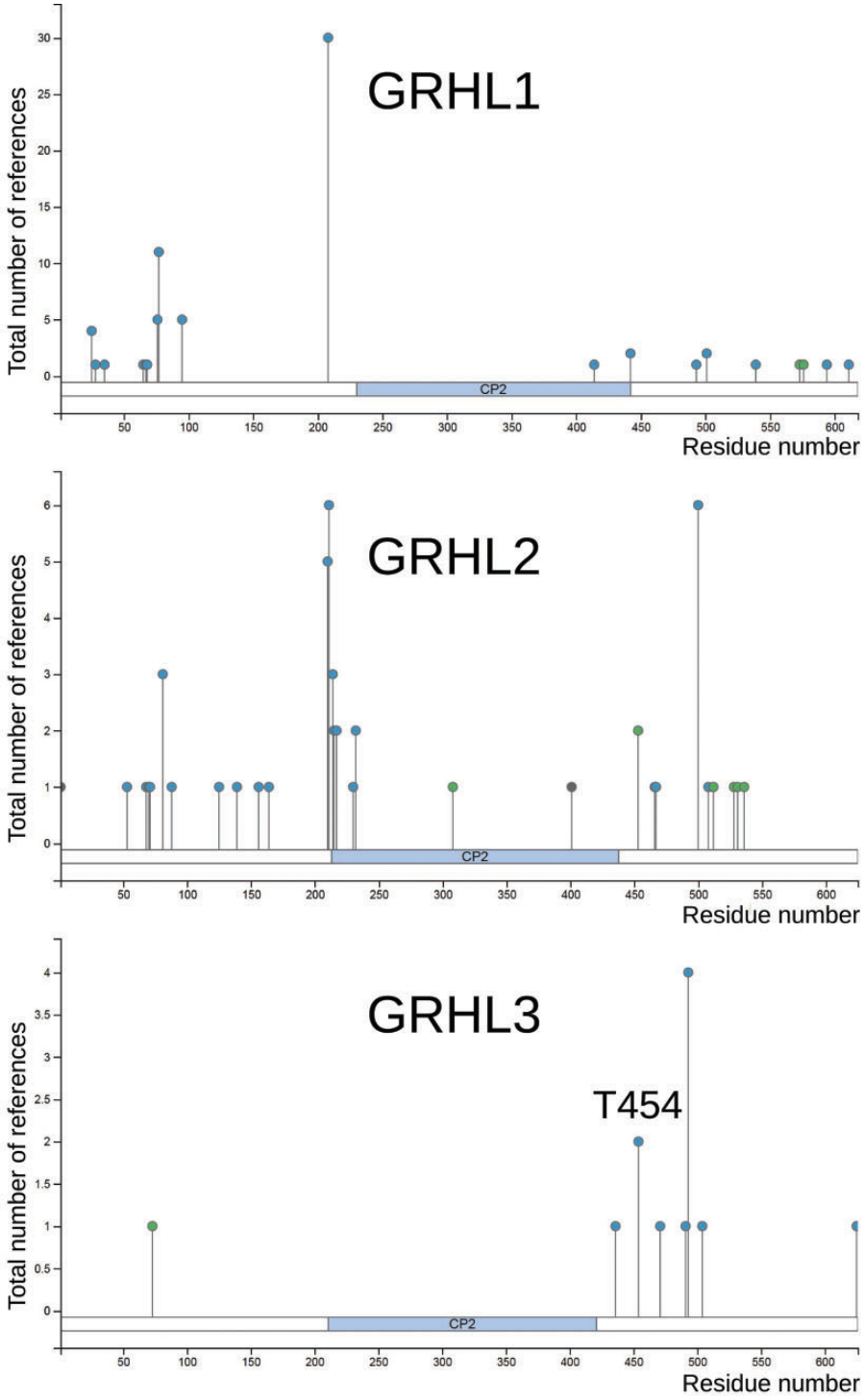

Figure 3. Posttranslational modifications of GRHL proteins. The data were obtained from the PhosphoSitePlus website (www.phosphosite.org, accessed October 2019).[47] Phosphorylation sites are indicated in blue, acetylation sites in green, others in grey. The location of the critical T454 phosphorylation site in the GRHL3 protein is marked above the bottom diagram.[46]

GRHL3 for use in treatment of those types of cancer in which these transcription factors perform tumor-promoting functions. There are known examples of transcription factor inhibitors, and some of them target late SV40 factor (LSF), which is closely related to the GRHL proteins.[42]

Polymorphisms in grainyhead-like genes associated with cancer susceptibility

Genome-wide association studies (GWAS) failed to identify germline polymorphisms in the *GRHL* genes that would be associated with the occurrence of any type of cancer.

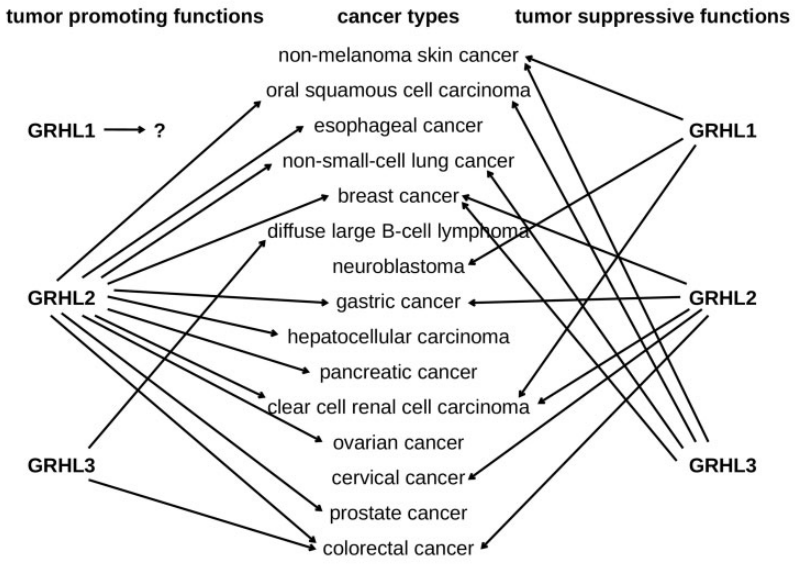

Figure 4. A summary of involvement of grainyhead-like transcription factors in cancer. This diagram was prepared on the basis of the present article and three recent publications.[1,3,34] Some results appear to be contradictory, with different publications reporting tumor promoting and tumor suppressive roles for the same GRHL factor in the same type of cancer. These discrepancies were extensively discussed in recent reviews.[1,3,4] A question mark indicates that no tumor promoting roles have ever been reported for GRHL1.

However, two studies from our laboratory targeted at the *GRHL* loci discovered single nucleotide polymorphisms (SNPs) whose presence was correlated with the incidence of either NMSC or ccRCC in human subjects.[33,34] The difference between the results of our investigations and GWAS can be explained by the major limitation of GWAS, which is underpowered to detect all the relevant variants as association signals must reach very high thresholds to be considered significant.[43] In total, we found 15 SNPs associated with ccRCC: 6 in the *GRHL1* gene, 6 in *GRHL2* and 3 in *GRHL3*.[34] All these polymorphisms were located in non-coding regions. In the case of NMSC, we detected six associated SNPs: five in *GRHL3* and one in *GRHL2*.[33] These are the only cancer-linked SNPs that have been identified in the *GRHL* genes so far. It is possible that the presence of these polymorphisms can allow to identify people with an increased risk of ccRCC or NMSC; however, studies of larger cohorts of patients are required to validate these SNPs as potential biomarkers.

Post-translational modifications of grainyhead-like proteins

In the studies described in the previous paragraph, we noticed two particularly interesting SNPs in the *GRHL3* gene, rs41268753 and rs141193530, which occur at statistically significantly altered frequencies in patients with NMSC.[33] Interestingly, in a different project, the same polymorphisms have been linked to nonsyndromic cleft palate, another disease associated with *GRHL3*.[44,45] These two SNPs are located in adjacent codons of the *GRHL3* gene, and the presence of either of them abolishes a putative phosphorylation site on threonine 454 residue (T454) in the encoded protein. The relevant protein fragment has the sequence PETDLE**TP**PVLFIP, where the affected amino acid residues are underlined. In individuals carrying SNP rs41268753, the encoded motif is changed to PETDLE**MP**PVLFIP, and in people carrying SNP rs141193530 it is changed to PETDLE**TA**PVLFIP. We therefore put forward a hypothesis that this TP motif is crucial for a very important phosphorylation event, and that the GRHL3 protein lacking this phosphorylation site cannot effectively perform its protective function against skin cancer and nonsyndromic cleft palate.

Our subsequent investigations have proven this hypothesis to be true.[46] TP is a characteristic motif recognized by proline-directed protein kinases. We confirmed that the T454 residue undergoes phosphorylation in human keratinocytes. This residue is very highly conserved among vertebrates. Substituting T454 with methionine, or substituting P455 with alanine, strongly decreased transcriptional transactivation activity of the GRHL3 protein. We discovered that T454 is phosphorylated by p38 mitogen-activated protein kinases (p38 MAPK), preferentially by p38α MAPK. Activation of p38 signaling in cells increases GRHL3 activity, and the T454 residue is important for the regulation of GRHL3 activity by the p38 MAPK pathway.[46]

We also found several additional phosphorylation sites in the GRHL3 protein.[46] More such sites were identified in proteomic discovery-mode mass spectrometry studies, the results of which have been deposited in public databases, such as PhosphoSitePlus (www.phosphosite.org, accessed October 2019).[47] In addition to phosphorylation, the GRHL proteins can also be acetylated at several residues. We summarize these findings in Figure 3. Post-translational modifications usually occur outside of structured domains; we noticed this trend in our earlier work.[46] However, with the sole exception of the T454 residue in GRHL3, none of these

modifications have been investigated in any detail. For this reason, the associated modifying enzymes are not known, and there is a lack of clear evidence that any of these post-translational modifications drive carcinogenesis. It is regrettable because it is very difficult to directly target transcription factors. An attractive alternative approach would be to target protein kinases that regulate the activity of GRHL transcription factors. Protein kinases are a "privileged" family of drug targets, as it is relatively easy to rationally design their inhibitors.[48] However, for this purpose, much more studies of post-translational modifications of GRHL proteins are needed in the future.

The role of grainyhead-like transcription factors in cancer is summarized in Figure 4.

Conclusions and future directions

In this article, we pointed out several aspects of involvement of GRHL transcription factors in cancer, which are often underappreciated by the scientific community.

The first such aspect is that the GRHL proteins in some situations perform oncogenic functions. Sometimes they display both tumor suppressive and oncogenic functions within the same type of cancer, and it is not clear how this is achieved.[4] Therefore, the underlying molecular mechanisms deserve further studies, as they may provide novel, valuable information about cancer development and potential new ways to combat this disease.

In those instances that the GRHL proteins perform tumor-promoting functions, they might be considered potential drug targets. However, it is difficult to directly target transcription factors, despite the development of some promising examples, such as inhibitors of LSF, a factor closely related to the GRHL proteins.[42] It is certainly much more feasible to design inhibitors of protein kinases.[48] In our earlier work, we presented evidence that the phosphorylation of threonine 454 is crucial for the transcriptional transactivation activity of the GRHL3 transcription factor.[46] It is thus a promising line for future research to investigate the phosphorylation of GRHL2, which acts as an oncogene in many types of cancer.[2–4] The GRHL2 protein can be phosphorylated at multiple sites (Figure 3), so the first task would be to determine which of these sites are relevant for the regulation of GRHL2 activity. Subsequently, it will be necessary to identify protein kinases that phosphorylate GRHL2 at these sites, then these kinases could become candidate drug targets.

Some single nucleotide polymorphisms in the *GRHL* genes may impair the functioning of these genes and thus contribute to the risk of disease development. Such examples have been found in the case of age-related hearing loss,[49] van der Woude syndrome,[50] and nonsyndromic cleft palate.[44,45] Our earlier research provided information about SNPs associated with the occurrence of ccRCC and NMSC in human patients.[33,34] The study of such polymorphisms is encouraged for two reasons. First, the presence of such SNPs may allow to identify people with an increased risk of certain types of cancer so that they could take preventive measures. Second, if at least some of these SNPs alter the functioning of the affected *GRHL* alleles, then studying them may shed new light on the mechanisms regulating the expression of *GRHL* genes or the functioning of GRHL proteins.

The study of somatic mutations in the *GRHL* genes in cancer samples is unlikely to lead to novel medical applications, as these mutations are widely distributed across the affected loci, and these mutations are very rare (Figure 2). Nevertheless, if driver mutations can be identified in this pool, then investigating molecular mechanisms responsible for their involvement in the disease development may prove beneficial as it may provide novel information about the functioning of *GRHL* genes and proteins, in the same way as the studies of disease-associated SNPs described in the previous paragraph.

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DECLARATION OF CONFLICTING INTERESTS

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