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Chapter 6

Concluding remarks
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Chromosomal instability is common in most tumor types, leading to copy number gains and losses of chromosomal regions. The high frequency of specific copy number alterations and epigenetic changes in each tumor type suggests that these alterations are beneficial for tumorigenesis, allowing tumor cells to acquire tumorigenic properties such as increased proliferation and evasion of apoptosis. The work performed in this thesis aimed to further investigate these changes, both in thyroid cancer (TC) and in colorectal cancer (CRC), with a focus on chromosome 7.

Retention of heterozygosity on chromosome 7 is seen in a diverse range of tumors, including carcinomas, sarcomas, and childhood malignancies such as neuroblastomas. This suggests that the molecular mechanism behind the retention of chromosome 7 heterozygosity is a potential Achilles heel for these different tumor types. The list of tumor types in which loss of heterozygosity on chromosome 7 is frequently observed is much shorter, namely adrenocortical carcinomas (ACC) and myeloid neoplasms.

In Chapter 2 we hypothesized that there is a set of mono-allelically expressed genes on chromosome 7 that are essential for tumor cell survival. To identify these possible “cancer cell survival genes” (CCSGs) we used oncocytic follicular TC (FTC-OV) as a model. These tumors have a remarkable genomic profile, with loss of heterozygosity of nearly all chromosomes occurring, with the exception of chromosome 7. The fact that FTC-OV show near homozygous genomes, and the fact that chromosome 7 is the only chromosome for which loss of heterozygosity is apparently not tolerated by the tumor cells, suggests that selection pressure precludes loss of heterozygosity on chromosome 7. Genome-wide DNA methylation profiling and allele specific expression analysis led to the identification of 6 imprinted CCSGs.

In addition to transcriptional regulation through DNA methylation, mono-allelic gene expression can also result from other mechanisms. For example, so-called expression quantitative trait loci (eQTLs) such as genetic variants in gene regulatory elements are associated with variation in gene expression (1). These variants can result in mono-allelic gene expression. Also stochastic effects can cause mono-allelic expression, as it has been described for the regulation of olfactory receptor genes (2, 3).

In order to further study the CCSGs on chromosome 7, we studied allele specific gene-expression in CRC tissues. Recent meta-analysis demonstrated that in CRC, like in TC, loss of heterozygosity of chromosome 7 is extremely rare (4). We used 21 novel CRC cell lines that were recently established in our laboratory. Cell lines provide a way to study more homogeneous tumor cell populations, with the potential of performing functional follow-up experiments on the same cell lines.

Before allele specific expression in the CRC cell lines was studied, they were thoroughly characterized (Chapter 3). The CRC cell lines were established with the aim of complementing the currently existing CRC cell lines, which have had several decades to adapt to culture conditions. Characterization of the novel cell lines included comprehensive investigation of somatic alterations such as mutations, changes in gene expression and genomic alterations. Using the obtained information, we were able to assess the most pronounced molecular alterations in these novel CRC cell lines, and the signal transduction pathways predominantly affected. Also, the allelic state of chromosome 7 in each of the cell lines was assessed. Additionally, as a result of the genomic characterization, we identified copy number alterations characteristic for liver metastases of CRC.

For our allele specific expression analysis
of the CRC cell lines we focused on allele specific expression on chromosome 7 (Chapter 4), with the aim to study the known and identify additional CCSGs. Our analysis identified several allele-specifically expressed genes on chromosome 7. Surprisingly, during validation of the results in a large cohort of colon and CRC samples, we observed allelic switching of three of these genes during tumorigenesis.

In Chapter 5 we integrated whole genome DNA methylation profiling and expression profiling of the CRC cell lines in order to assess the contribution of DNA methylation to transcriptional (down)-regulation in the cell lines. In light of the findings in Chapter 2, we also analyzed the chromosome 7 imprinting clusters in the CRC cell lines. We found that unlike in TC, in CRC cell lines the imprinting control regions were not always hemi-methylated. Notably, nearly all sites identified in Chapter 2 which were not hemi-methylated in the cell lines were hypermethylated. The latter observation is in concordance with what we found in the ACC samples tested, as discussed in Chapter 2. The ACC tumors with LOH of chromosome 7, which were used as controls, displayed high methylation levels at the chromosome 7 imprinting clusters identified in FTC-OV. This indicated that either retention of the methylated allele, or hypermethylation occurs in the ACC and CRC samples.

The analyses in Chapter 5 focused on combining the gene expression data generated for Chapters 3 and 4 with DNA methylation data. The analysis revealed extensive DNA methylation in CRC carrying a BRAF mutation, resulting in downregulation of genes involved in amongst others apoptosis and lipogenesis.

Mechanisms of chromosomal instability in cancer

As discussed, the genome wide near-haploidisation with the retention of heterozygosity on chromosome 7 is striking in FTC-OV. The latter resulted in our initial studies into the mechanism behind the retention of chromosome 7 in TC. We hypothesized that when loss of chromosome 7 would occur such an event would be catastrophic for those particular TC cells in which this has happened. However, to support our hypothesis of selection pressure being exerted, losses of chromosome 7 should be proven to occur. We did however not test whether losses of chromosome 7 actually occur in TC.

A whole chromosome instability profile observed in FTC-OV has been suggested to be either the result of mis-segregation events occurring during mitosis, or alternatively of meiosis-like events (5, 6). Analysis of multiple fractions of the same tumour would already suggest a gradual loss of chromosomes. In recent literature multiple genes and pathways have been implicated in chromosomal mis-segregations in tumor cells, including DNA damage, mitotic spindle assembly and long noncoding RNAs (7-9).

Numerical chromosomal aberrations are shown to be increased in cells with active DNA damage response signalling (10). Activation of the DNA damage response checkpoints; also in the absence of DNA damage, has been shown to result in over-stabilization of the kinetochore-microtubule attachment, leading to chromosomal mis-segregations (11). Alternatively, reduced function of spindle assembly checkpoint proteins BUB1 and BUB3 was shown to lead to high frequencies of numerical chromosomal changes, even in normal tissues (12).

The most recent addition to the list of potential causes of a whole chromosome instability profile as seen in FTC-OV is the role of the long noncoding RNA NORAD. This long noncoding RNA is highly conserved and ubiquitously expressed.
in all cells. NORADs main function is sequestering PUMILIO proteins, preventing these proteins to bind mRNAs. PUMILIO proteins bind a wide range of mRNAs involved in homeostasis and cell cycle control. In absence of NORAD PUMILIO proteins bind these mRNAs, reducing their stability and inhibiting translation. The latter was shown to result in aneuploidy, as well as endoreduplication (8).

**Dynamics of allele specific expression**

Analysis of the DNA methylation data showed that the chr7q32.2 imprinting cluster is fully methylated in our primary CRC cell lines, and also in the colon adenocarcinoma samples from The Cancer Genome Atlas (13). This limits the number of genes at this imprinting cluster that could play a role in the retention of heterozygosity on chromosome 7 in CRC to only those that are normally expressed from the maternal (methylated) allele. To further investigate additional genes precluding loss of heterozygosity on chromosome 7 in CRC, allele specific expression and siRNA experiments should be performed. For these experiments, the CRC cell lines described in Chapter 3 offer a valuable model, as these are amongst the best characterized CRC cell lines in existence.

In CRC allele specific gene expression analysis revealed allelic switching of DLX5, GRB10 and SVOPL when comparing tumors with matching normal mucosa. DLX5 is a member of the DLX family of homeodomain transcription factors, which share sequence similarities with the Drosophila distal-less gene. DLX5 has been shown to stimulate tumor cell proliferation by upregulating MYC expression (14, 15). GRB10 encodes a growth-receptor bound protein that plays an important role in multiple key cancer signalling pathways such as Wnt and Akt signalling (16, 17). Therefore DLX5 and GRB10 are putative oncogenes, and the allelic switching could serve as a mechanism to compensate for transcriptional silencing of the expressed allele, or switch to the alternative allele when the expressing allele is mutated. The potential role of SVOPL in tumorigenesis so far remains unknown. SVOPL stands for SVOP-like protein, as it is a paralog to the synaptic vesicle protein SVOP. SVOPL is marked as a putative transporter, but no substrates have been identified so far (18).

Similar to our findings, in lung cancer pro-apoptotic factor BCL2L10 has been shown to display switching of the most abundantly expressed allele during tumorigenesis (19). In case of SVOPL, transcriptional downregulation was observed in all paired CRC samples compared to the normal mucosa. This suggested that the allelic switching observed for SVOPL is primarily the result of downregulation of the most abundantly expressed allele, possibly in conjunction with a slight upregulation of the other allele. For DLX5 and GRB10 this was not the case. Therefore we concluded the allelic switching of these genes must be due to a change in transcriptional regulation, affecting both alleles differently.

To definitively prove the allelic switching occurs during tumorigenesis, more research is required. One alternative explanation is mosaicism, where there are patches of a distinct population of cells within an individual (20, 21). If the CRC arises from one of these distinct patches, and the adjacent normal mucosa is isolated outside of that patch (or vice versa) the test results could suggest allelic switching. A recent publication identified mosaic somatic events in the blood of 14% of the test population (22). Such high frequency of mosaicism means that mosaicisms should really be considered as a cause for the observed allelic switching. We do however note that mosaicism leading to gene regulatory effects have so far not been reported in the literature. It is furthermore not inconceivable.
that mosaicism would arise before, or at the same time as the establishment of the allele specific expression of DLX5 and GRB10 thereby resulting in something as gene-regulatory mosaicism.

More likely the allele specific expression status of DLX5 and GRB10 could be determined in each stem cell separately, giving rise to groups of cells all expressing one particular allele. If the latter were the case, the cells that eventually lead to tumor formation would randomly have 50% chance of expressing the genes from the same allele as the tissue that is sampled for normal colon mucosa. Additionally, we would expect bi-allelic expression to occur more often in the normal mucosa when compared to CRC. The normal samples contain many different crypts, whilst the CRC are expected to originate in most cases from one clonal crypt cell. Both mosaicisms and stem-cell specific allele specific expression regulation can still not be excluded as a cause for the allelic switching of DLX5 and GRB10.

The mechanism that resulted in the allelic switching is unclear, as well as the effect that it has on tumor cells. Extensive allele specific expression effects, where one allele is completely or nearly completely silenced, can be regulated either epigenetically or by conformational changes of the DNA. The most well-known examples of epigenetic mono-allelic silencing are imprinting and X-chromosome inactivation (23, 24). During cell division DNA methylation is subsequently copied to newly synthesized DNA strands, ensuring stable continuation of the allele specific expression. For DNA methylation to regulate the allelic switching, DNA methylation would need to be switched from one allele to the other. At the frequency the allelic switching occurs such a scenario seems unlikely. Alternatively, structural changes in the three dimensional chromosomal organisation could potentially result in an extensive transcriptional deregulation such as allelic switching. Three dimensional chromosomal structures can range from large-scale folding of whole chromosomes to chromosomal looping connecting promoters to enhancer elements in cis (25). Chromosomal loops regulate gene expression by bringing together transcriptional enhancers or repressors, or by separating them. Alternatively, tethering of the DNA to the nuclear lamina by proteins such as CCCTC-binding factor (CTCF) and cohesin can result in gene silencing as they are withheld from the nucleolar periphery, where most of the transcription factories are located (26, 27). All such events can potentially affect large sets of genes, located both in cis and in trans.

**Final conclusions**

In TC we hypothesized that selection pressure would prevent loss of heterozygosity on chromosome 7 and we identified 6 imprinted CCSGs allele specifically expressed from one of the alleles of chromosome 7. These CCSGs, and the molecular and cellular pathways they are involved in seem to be essential for TC tumour cell survival. Therefore the CCSGs are potential therapeutic targets for the treatment of FTC-OV, a TC subtype that often shows recurrence due to failure to respond upon radioactive iodine (RAI) treatment. RAI treatment is one of the most effective treatments in thyroid tumour cells that accumulate iodine. Secondly we found that the identified CCSGs in TC are possibly less relevant in adrenocortical carcinomas and in CRC. The survival genes identified in Chapter 2 seem to be more tissue and disease specific than originally anticipated. Similar to ACC in CRC, the loci with cell survival genes were frequently hypermethylated instead of the expected hemi-methylation. Furthermore we came across extensive allelic switching in CRC. The occurrence of allelic switches in drugable gene targets might potentially
interfere with treatment responses. In order to understand the mechanism of allelic switching, chromosomal conformation studies should be performed studying a wide variety of genes.

REFERENCES


Concluding remarks


