# Next generation sequencing of ovarian metastases of colorectal cancer

Crobach, A.S.L.P.

# Chapter 7

# Concluding remarks and future directions

This thesis describes the application of next-generation sequencing to the optimal diagnosis of ovarian tumors. For ovarian tumors, knowledge about their tissue of origin is crucial for therapeutic choices and statements regarding prognosis. The origin can be difficult to determine in a subset of cases.[1, 2] Macroscopic and histologic examination complemented by immunohistochemistry can, in some situations, be insufficient to identify the origin of a tumor process.[3, 4] Antibodies for novel targets are being developed but immunohistochemistry can still be difficult to interpret, not adhering to unmistakable guidelines. Therefore, additional (molecular) techniques are needed to help identify the origin of a tumor.

In chapter two, we describe a series of colorectal cancer (CRC) and duodenal cancer cases with ovarian metastases. In a cohort of 30 ovarian CRC metastases, four patients with Familial Adenomatous Polyposis (FAP) were identified. This enrichment of FAP patients in a series of CRCs is striking since the estimated incidence of FAP CRC is far below 1% of all CRCs.[5] The expected incidence of FAP CRC that metastasizes to the ovaries would thus be almost zero. The necessity of combined surgical resection of the primary CRC and bilateral oophorectomy is a matter of debate. Especially in female FAP CRC patients, bilateral oophorectomy during surgery should be discussed.

In chapter 3, we used a custom-made next-generation sequencing (NGS) panel, including 115 cancer-driving genes to screen formalin-fixed paraffin-embedded (FFPE) tumor tissue from 43 primary endometrioid and mucinous ovarian carcinomas. We then compared them to 28 proven ovarian colorectal cancer (CRC) metastases. The mutations were validated by high-resolution melting curve analysis (HRMCA) and Sanger sequencing. Furthermore, loss of heterozygosity (LOH) and promoter hypermethylation of APC were also studied. In the primary ovarian tumors, *TP53*, *NOTCH1*, *PIK3CA* and *FAT4* were the most frequently mutated genes. In the ovarian metastases, *APC*, *TP53*, *KRAS* and *FAT4* mutations were the most common mutations in ovarian CRC metastases. Inactivating *APC* mutations were identified in 71% of CRC metastases, which is in contrast to 4.7% identified in primary ovarian tumors. LOH and APC promoter hypermethylation did not differ significantly between the primary and secondary ovarian tumors. It can be concluded that *APC* mutation analysis can be used to differentiate primary endometrioid and mucinous ovarian tumors from ovarian CRC metastases.

In chapter four, the gene panel from chapter 3 was used to analyze the mutational profiles of primary colorectal cancers (CRCs) and the corresponding ovarian metastases. We compared 26 primary CRCs and 30 matching ovarian metastases (4 with

bilateral metastases). Low thresholds were used in bioinformatics analysis to prevent low frequency passenger mutations from being filtered out. Sanger and/or hydrolysis probe assays were used to validate a subset of variants. No striking differences were observed between the mutational landscape of CRC that metastasized to the ovary and CRC in consecutive series.[6] There was considerable overlap in the mutations of early-affected genes when comparing primary CRCs and their matching ovarian metastases. A subset of mutations, presumably passenger mutations, demonstrated less overlap. In particular, primary CRCs showed a substantially high number of supposed passenger mutations. We also compared the primary CRCs and matching metastases for stratifying variants of 6 genes (*KRAS*, *NRAS*, *BRAF*, *FBXW7*, *PTEN* and *PIK3CA*) that select for established (*EGFR*-directed) or future targeted therapies. Out of a total of 31 variants, 12 were not found in either of the two locations. Therefore, the number of discordant variants between the primary tumors and their matching metastases differed. Half of these discordant variants were pathogenic variants. In terms of temporal heterogeneity, no clear relationship was observed between the number of discordant variants related to the time interval between primary CRCs and the detection of ovarian metastases. Dormant metastases may therefore be present from the early days of the primary tumors.

In chapter 5, the Haloplex targeting technique used for next-generation sequencing (NGS) was studied and validated with another amplicon based targeted approach (Ion Ampliseq).[7, 8] NGS has been proven to be successfully applicable to fragmented formalin-fixed paraffin-embedded (FFPE) tissue.[9] A large number of targeted sequencing approaches are offered based on different principles, such as polymerase chain reaction (PCR), hybridization or circularization.[10] We showed that a circularization-based approach (HaloPlex), followed by sequencing on Illumina HiSeq, is successful for targeted sequencing of DNA from FFPE material. Detected variants were validated with a PCR-based targeted enrichment method (Ion AmpliSeq), followed by sequencing on an Ion PGM sequencer. A high concordance rate between the detected variants from different sample preparation techniques and sequencing methods was observed. The discordant variants could largely be explained by (subtle) setting differences in the analysis pipeline. Thus, optimal bioinformatics analysis is crucial for the correct detection of variants. In addition, tumor intra-heterogeneity (ITH) resulting in different DNA isolates can cause discordant sequencing results.

The case report in chapter 6 describes a female patient suspected of having Lynch syndrome. She was diagnosed with bilateral ovarian cancer at age 44, followed by the detection of a colon carcinoma 12.5 months later. Patients with synchronous or

metachronous ovarian and colon cancers pose diagnostic challenges.[11] Primary colon carcinomas can metastasize to one or both ovaries, two independent primary tumors can arise or an ovarian carcinoma can metastasize to the colon. Clinical and immunohistochemical characterization can be helpful. In this case, lesions of both sites showed a DNA mismatch repair deficiency with immunohistochemical expression loss of MLH1 without *MLH1* promoter hypermethylation. In the absence of germ line MMR gene variants, identical somatic *MLH1* and *CTNNB1* gene variants were found, indicating a clonal relation. MMR germ line mosaicism was ruled out by ultra-deep sequencing of the *MLH1* variant in DNA isolated from normal mucosa, blood, urine and saliva. Although initially suspected of having Lynch syndrome, it was eventually concluded that a metachronously metastasized colorectal tumor to both ovaries was most likely. This report illustrates the diagnostic dilemmas that can be encountered in solving suspected Lynch syndrome cases.

## Next-generation sequencing (NGS)

The last decade brought enormous technical developments in the field of molecular diagnostics. Sanger sequencing, a labor-intensive technique in which limited regions of a gene can be analyzed, has been replaced by next-generation sequencing (NGS), in which multiple genes can be examined in parallel.[10, 12] Next, the development of targeted therapies directed at specific genetic variants has taken an important place in the treatment of oncologic patients.[13] The detection of specific genetic variants is decisive in choosing the optimal treatment strategy. With extensive molecular profiling, the number of molecular targets that can be treated by specific agents is increasing. Therefore, accurate and fast methods are needed to detect targetable variants.[14]

Currently, targeted techniques in which only a selection of genes is investigated is the preferred method in a clinical setting, as the gene targeting and the subsequent bioinformatic analysis is less complex.[12, 15] Still, targeted sequencing devices that decrease sequencing time and reduce costs are desired. Most likely, whole-genome approaches will eventually become more important in the future.[16] A complete overview of most genomic variants, without first identifying the DNA of interest, can be obtained in this way.

Currently, several sequencing platforms are available, all of which have their pros and cons.[12] The development of these platforms resulted in the generation of longer sequencing reads (now generally several hundreds of base pairs), making genome assembly easier. A recent development in the field of NGS is the generation of even longer reads (thousands of base pairs).[12] These long reads are helpful in analyzing repetitive elements and complex structural variants.

The recent single molecule sequencing platforms overcame the problems generated by amplification of the input DNA.[12] As very limited input material is needed and the sequencing speed is faster than in the existing techniques, single molecule sequencing holds great potential for the future.[17] This is especially true as the input of tumor DNA might be decreasing with the increased use of neoadjuvant therapies (e.g., breast, rectum, esophagus and stomach cancers). Additionally, the applicability of sequencing techniques with freely circulating plasma-derived DNA as input, which will become more important in the near future, also needs to be investigated. Finally, the focus of attention may be shifting to sequencing non-coding regions, as recent research has shown that non-coding regions are involved in carcinogenesis.[18]

## Bioinformatic analysis

NGS produces enormous amounts of data. Bioinformatics analysis is necessary to obtain trustworthy data.[19] A list of pathogenic variants should not be contaminated by false-positive or false-negative findings. False-positive findings can be caused by technical issues that can differ by platform. Another problem is deciding whether a variant is non-pathogenic and present in the population or whether it is a true pathogenic variant responsible for tumor development. False-negative findings can be caused by suboptimal filter setting in a bioinformatic pipeline. As whole-genome sequencing is becoming more important, bioinformatic analysis and data storage infrastructure have to be able to cope with this increasing amount of data.[16] Next, new sequencing platforms can offer benefits with respect to the speed and the cost of sequencing. However, specific artifacts potentially linked to new sequencing techniques must be examined.

The refinement of sequencing detection techniques leads to problematic decision making about thresholds. The previously used Sanger sequencing was much less sensitive than the current NGS techniques. With Sanger sequencing, a somatic genetic variant was detectable once it was present in lesions with >20% of tumor cells. With the current techniques, somatic variants present in 0.1% of the cells can be detected. Whether the variant profile of such a small percentage of tumor cells should be decisive in choosing treatment strategies is unknown. For example, if 0.1% of the tumor cells of a colon carcinoma carry a *KRAS* mutation, it is unknown whether that patient should be excluded from targeted therapy or if it is still effective to target the other 99.9% of the tumor cells.

A positive effect of the high sensitivity of NGS techniques is the ability to detect variants in samples with a low tumor percentage. Previously, variants were often not discovered in samples with only a limited number of tumor cells. However, whether these

negative findings were false-negative observations could not be determined. Currently, the absence of variants after NGS analysis with sufficient read depth can be stated with much more certainty.

However, with a low input of DNA, the number of separate DNA molecules is also reduced. Sequencing artifacts caused by the NGS protocol, due to, for example, preferential amplification or cytosine deamination, can result in false-positive and false-negative results. Single molecule molecular inversion probes (smMIP) can help with detecting those sequencing artifacts.[20, 21]

## Defining the origin of tumors

With the introduction of NGS, the generation of tumor-specific mutation profiles, correlated to the primary tumor site, was anticipated. If each tumor type showed a characteristic mutation profile, these profiles could be used to define tumor origins. However, extensive sequencing projects (e.g., the TCGA project) have revealed that most tumor types share similar driver variants, in addition to a wide spectrum of passenger variants that are present in subclones within the tumor [6, 22]. In general, there are no specific mutation profiles linked to the origin of a tumor. However, according to the clinical setting, variants in specific genes can be informative.[23]

Somatic mutation detection is only one component of the molecular profile of a tumor. Copy number alterations can be detected by NGS or by array technology.[24]

Several array approaches that can help identify the primary location of a tumor have been described. Some of these tests are commercially available.[25] A more functional read-out of somatic variants and gene fusions can be obtained by transcriptome sequencing that covers the complete set of RNA proteins. Finally, epigenetic modifications influencing the expression levels of genes could be informative. Integration of all these techniques will produce a more complete molecular profile of tumors. Most likely, better predictions can be made about the origin of cancer metastasis in cases of unknown primary tumors based on these integrated profiles.

Nevertheless, despite the application of currently available tools (imaging, immunohistochemistry, and molecular analysis) approximately 3% of carcinomas cannot be traced back to their origin.[26] One may argue that defining the origin may be less important as potential targeted therapies can be applied irrespective of the location of the tumor (“basket studies”). The effectiveness of such an approach is currently being investigated. However, molecular analysis should be considered in the context of the complete clinical picture. For instance, *BRAF* inhibition alone can be an effec-

tive treatment for disseminated melanoma, but not for a metastasized colon carcinoma.[27]

### Targeting therapies

Clinical trials have shown that targeting a specific genetic variant can result in spectacular results. However, the effect of these therapies often only lasts for a limited period (several months). The idea that the targeting of (e.g., inhibition of) a single driver gene of a tumor could be enough to stop the growth of a malignant process has been proven to be too simplistic. Escape mechanisms are activated in tumor cells that enable their continuous growth.[28] A combination of targeted therapies aimed at more than one pathway has been shown to be more effective. For example, a combined therapy of *BRAF*- and *MEK*-inhibition in melanoma with *BRAF* mutations showed an improved response in comparison with *BRAF*-targeted single therapy.[29]

### Intra-tumor heterogeneity (ITH)

Another challenge with targeted therapies is that the genetic variants that are targeted can be present in only a portion of the tumor cells, reflecting intra-tumor heterogeneity (ITH). Tumors arise initially from a single cell in which driver mutations create a growth advantage. Further progression of the tumor leads to the accumulation of additional mutations, which are present in subclones of the tumor. ITH develops over time (temporal heterogeneity) and space (spatial heterogeneity), leading to a tumor consisting of a heterogeneous population of cells.[30] Targeted therapies that are directed against specific genetic variants will not be effective in tumor cells not carrying those specific variants. To get an up-to-date and complete overview of the genetic changes within a tumor, multiple samples over time and from different regions should be taken. Such an approach is impractical. Currently, the choices of targeted therapies are based on a single biopsy at one moment in time. That molecular profile is not an up-to-date and complete reflection of the mutation profile of the tumor. Liquid biopsies (the analysis of tumor DNA in systemic circulation) may overcome these problems. However, at this moment, it is not known whether the subclones that are present in the tumor contribute equally to circulating DNA.

Although targeted therapies may so far not have resulted in effective and sustained results in tumors, personalized care will remain an important treatment module in oncology. Standard use of liquid biopsies, simultaneous treatment with multiple drugs and expanding the repertoire of agents directed to targetable variants will be seen in the near future. Another important development in cancer treatment is immunotherapy.[31] Immunotherapy can be categorized into two types. The first type is active immunotherapy, in which the immune system of the patient is directed to the tumor

cells. The second type, passive immunotherapy, involves enabling existing immune responses to attack tumor cells or injecting antibodies or T-cells.

An active response of the immune system directed to the tumor cells can be achieved by injecting tumor specific antigens, as is done with the BCG (Bacillus Calmette-Guérin) treatments for bladder cancer. Antigen vaccines are now also developed for, among others, melanoma, colon cancer and leukemia.

Administration of antibodies is now possible for certain forms of lymphoma (anti-CD20), colorectal carcinoma (anti-EGFR / anti-VEGF) and gastric/breast carcinoma (anti-Her2neu).

Another option to create an anti-tumor response by the immune system is the injection of activated dendritic cells expressing tumor antigens.[32] These dendritic cells will provoke an immune response in lymph nodes. Successful trials have been performed for melanoma, renal cell carcinoma, lymphoma and glioblastoma.

Tumor infiltrating lymphocytes (TILs) are often present in tumor tissue but are not very effective because of the immunosuppressive environment that is created by the tumor cells.[33] Harvesting TILs from a patient, expanding their numbers, activating them and returning them into the patient can result in a tumor cell-directed immune response. Immunomodulation is possible by anti-CTL antigen-4 (CTLA-4) antibodies that neutralizes the inhibiting activity on T-cells.[34, 35] Anti-PD1 antibodies have a comparable working mechanism.

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