Tumor evolution and intratumor heterogeneity in colorectal carcinoma: insights from comparative genomic profiling of primary tumors and matched metastases

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Abstract: Metastatic colorectal cancer (CRC) is one of the leading causes of cancer-related mortality among men and women worldwide. Over the past few decades, advances in our understanding of the genetic and epigenetic underpinnings of CRC have led to important insights into the pathogenesis of invasive tumors and have identified different molecular subgroups. Nonetheless, the events that might facilitate dissemination of tumor cells to distant sites giving rise to metastatic disease are not well characterized. Furthermore, in contrast to intertumor heterogeneity the extent of intratumor heterogeneity in different types of CRC has not been fully defined. In this paper, we review studies that have compared the genetic profile of primary invasive carcinomas to that of matched metastases and discuss the implications of their findings for our understanding of tumor evolution and for the clinical management of patients with advanced CRC.

Keywords: Colorectal carcinoma; intratumor heterogeneity; tumor evolution

Introduction

Colorectal cancer (CRC) is the second most common cause of death in the United States with over 50,000 deaths estimated in 2014 (1). Most deaths result from complications associated with metastatic disease, which affects approximately 35% of patients. Clinically and histologically, most invasive tumors arise from adenomas or sessile serrated polyps, which can evolve over many years if they are not removed. In their seminal 1990 paper Fearon and Vogelstein proposed a tumor progression sequence based on which invasive carcinomas arise from adenomas via the sequential accumulation of genetic changes (2). Most commonly, the initiating event is a mutation in the *APC* gene, which results in activation of the WNT/beta-catenin pathway. Mutations that constitutively activate the RAS/RAF/MAPK pathway promote tumor growth and adenoma formation, while subsequent clonal expansion and the transition from adenoma to invasive carcinoma results from alterations in genes such as *TP53*, *SMAD4* or other components of the TGF-beta pathway (3). In the majority of cases, the progression from adenoma to carcinoma is associated with chromosomal instability, most notably gains in chromosomes 8q, 13q and 20q and losses encompassing the 1p, 8p, 17p and 18q chromosomal regions (4). Sessile serrated polyps progress to invasive carcinomas through a different pathway characterized by microsatellite instability and CpG island methylation rather than chromosomal instability (5).

Some primary invasive tumors acquire the ability to spread to other organs, and patients may either present with metastatic disease or develop distant metastasis after resection of their primary tumors. The genetic and epigenetic mechanisms that mediate the metastatic spread in individual patients are not as well understood as those events that occur early in CRC pathogenesis and this has hampered the development of effective, targeted approaches that can prevent and treat metastatic disease. Also limited are insights into the intratumor heterogeneity of advanced CRC, which can have practical implications for clinical care. For example, a consensus does not currently exist as to whether analysis of the primary tumor is sufficient or whether a metastatic lesion should be studied in patients with metastases. In this paper, we review studies that have compared the genomic profiles of matched primary CRC tumors and metastases and discuss their findings in the context of tumor evolution and clinical practice.

Comparative genomic hybridization (CGH) studies

Initial studies investigating the relationship of metastases to primary tumors used genomic hybridization techniques to compare the chromosomal abnormalities in matched pairs. In one of the earliest studies, Korn et al. reported that liver metastases showed chromosomal changes similar to those observed in primary tumors (6). They did not find any consistent differences between matched pairs with the exception of a gain of chromosome arm 11q, which was identified in two of six liver metastases but not in any of the matched primary tumors. In a similar study using CGH to examine aberrations in 12 matched pairs, Al-Mulla et al. found gains in chromosome arms 7p, 8q, 13q and 20q and losses of chromosomes 4 and 18 and chromosome arm 8p to be more common in metastases, while the most frequent genetic change associated with metastasis was deletion of chromosome 22 (7). Primary tumors and their synchronous metastases shared the same copy number alterations in all but one case, supporting a clonal relationship. Nonetheless, they were never identical, consistent with the presence of heterogeneity between primary tumors and metastases. In all patients, the metastasis had acquired new genetic alterations not found in the primary tumor, although the number of genetic alterations was not always higher in the metastasis.

Subsequent studies have largely confirmed these findings. Aragane et al. reported a high concordance of chromosomal alterations between primary tumors and liver metastases (8).

Metastases had a higher number of aberrations and displayed more frequent loss of chromosome arm 18p and gains at 8q and 20q when compared to the primary tumors. In another study, Alcock et al. performed CGH on 17 matched pairs of primary tumors and liver metastases and found that the majority (15/17) were clonally related. Every sample, however, had unique changes not shared by the matched sample, which the authors speculated was the result of random genetic changes superimposed on multiple waves of clonal expansion over time. In that study the only genetic event that was significantly associated with metastases was loss of 4q32-34 (9).

Loss of chromosome arm 4q was also noted as being more frequent in metastases in a study by Jiang et al., where investigators examined the relationship between 18 primary tumors and their matched pulmonary metastases (10). In this study, all pulmonary metastases were found to have more chromosomal alterations than the primary tumors. Ten cases (56%) exhibited a high degree of clonal relatedness, however the remaining eight cases showed significant discordance, suggesting that the metastatic clone either represented a distinct clone or underwent many additional chromosomal changes upon dissemination. The higher degree of discordance seen in this study of pulmonary metastases compared to prior studies of liver metastases raises the possibility that the extent of heterogeneity varies among different metastatic sites.

Knösel et al. included both lymph node metastases and systemic metastases in their study and found differences both between metastases and matched primary tumors and between different types of metastases (11). Specifically, lymph node metastases had more frequent deletions of chromosomes 18 and 21q compared to primary tumors, while liver metastases had an even higher number of alterations characterized by deletions at chromosomal arms 2q, 5q, 8p, 9p, 10q, 11p and 21q and gains at 1q, 11q, 12qter, 17q12-21, 19 and 22q compared to primary tumors. Based on these findings, the authors suggested that lymphatic and hematogenous tumor spread represent independent pathways to colorectal tumor dissemination.

More recently our group performed array CGH on 25 matched primary tumors and liver metastases (12). We found that liver metastases harbored more chromosomal alterations compared to primary tumors (9.6% vs. 7.5%) but did not observe a recurrent focal area of copy number change in metastatic samples that was not present in the primary tumor. Clonality analysis classified 22 pairs (88%) as clonal. In two of the three cases that were not clonal

by our analysis, the patients had two primary tumors suggesting that the metastasis was derived from a different primary. In the third case, the patient had received radiation of his primary rectal tumor following resection of the liver metastasis, suggesting the possibility that localized radiation may have significantly altered the copy number profile of the primary tumor.

Taken together, CGH studies have shown a high degree of genetic similarity among matched primary-metastasis pairs, indicating dissemination of a clone within the primary tumor. The significant discordance seen in a minority of cases might be secondary to the presence of multiple primary tumors or localized radiation. Importantly, even when clonally related, primary tumors and metastases are not identical. Metastases appear to commonly harbor additional alterations when compared with the primary tumor and sometimes lack alterations detected in the primary tumor, suggesting ongoing chromosomal instability both in the metastasis and in the primary tumor following dissemination of the metastatic clone. Metastatic site might be associated with the level of chromosomal instability, but more studies using tissue form extrahepatic metastases are needed to address this possibility.

Recurrent metastasis-specific alterations identified in the CGH studies included gains in chromosome arms 8q, 11q and 20q, along with losses in chromosomes 4, 8p and 18. Chromosome arms 8q and 11q contain the well-characterized oncogenes C-*MYC* and *CCND1* genes, respectively. Chromosome 20q contains the *MMP-9* gene, whose overexpression has been linked to a more invasive phenotype *in vitro* and to reduced disease-free survival in CRC patients (13,14). No defined tumor suppressor genes have been identified on chromosome 4, whereas several potential possible tumor suppressors genes have been localized to arm 8p, indicating it may be a hot-spot susceptibility locus in the progression of CRC (15,16). Tumor suppressor genes *DCC*, *SMAD4* and *SMAD2* have all been mapped to chromosome 18, and loss of these genes has been linked to CRC progression in some studies (17-19).

Early sequencing studies

Investigations of the mutational profile of metastases in comparison to primary tumors have focused largely on the *KRAS* gene, because of its central role in routine clinical practice. Determination of the *KRAS* mutational status is required when patients are being considered for treatment with either cetuximab or panitumumab, both of which

are monoclonal antibodies that bind to the epidermal growth factor receptor (EGFR) (20-22), as these drugs are ineffective in patients whose tumors harbor *KRAS* mutations (23-25). Given that patients with metastatic disease have multiple sites involved by tumor, defining the extent, if any, of heterogeneity in the *KRAS* mutational status among different sites can guide tissue selection for clinical molecular testing.

To date, the majority of studies in patients who have not received anti-EGFR therapy have found a very high concordance in the *KRAS* mutational status between primary tumors and metastases, and this is consistent with *KRAS* mutations being an early event in CRC tumorigenesis. Etienne-Grimaldi *et al.* reported 100% concordance of *KRAS* mutations between primary CRC and matched liver metastases in a study of 93 patients, in which 39% of them had a mutation in either KRAS codon 12 or 13 (26). Similarly, Santini *et al.* reported a 96% concordance rate in 99 matched primary tumors and metastases that included lesions from the liver (n=80), lung (n=7), and peritoneum (n=5), as well as other sites (27). Among the four discordant cases encountered in that study, a *KRAS* mutation was found in a metachronously resected peritoneal metastasis of a patient with a wild-type primary tumor. In the remaining three cases, the primary tumors showed a mutant *KRAS* allele, whereas the liver metastases were wild-type.

Conflicting results have been reported in some smaller studies (28,29). For example, in a study of 28 matched primary tumors and lymph node metastases looking both at *KRAS* and *BRAF* mutational status, Oliveira *et al.* reported a high discordance rate of 36% (28). In the ten discordant cases reported, five patients whose primary tumor was wild-type had a mutation in the metastasis, while in the remaining patients a mutation was identified in the primary tumor that was not seen in the metastasis. In that study, a significant number of tumors had both a *KRAS* and a *BRAF* hotspot mutation—a finding that raises concerns about the validity of the sequencing results, as mutations in *KRAS* and *BRAF* tend to be mutually exclusive in CRC. Moreover, false-negative results are more common when analyzing tissue from lymph node metastases due to low tumor purity.

Our group examined a cohort of 84 matched primary and metastatic pairs and also found high (>92%) concordance rates for mutations found in *KRAS/NRAS*, *BRAF*, *PIK3CA* and *TP53* (12). In our study using a mass-spectrometry based assay, we first assessed the *KRAS* mutational status using frozen tissue and found it to be concordant in 78/84

(92.8%) of matched pairs. The concordance rate rose to 97.6% (82/84) after we tested formalin-fixed paraffin-embedded (FFPE) tissue from discordant cases. This highlights the fact that false-negative results can be observed in some cases, most likely due to technical reasons such as low tumor purity. *KRAS* mutation status was discordant in two cases after examination of both frozen and FFPE tissue, and in both of these cases there was clinical suspicion of a second primary tumor. The lowest rate of concordance encountered in our study was 92.8% and was seen in *TP53* where there were six discordant cases. In three of these cases, the discordance was attributed to the *TP53* mutation found in the liver metastasis being absent from a superficial/pre-invasive portion of the primary tumor, though it was present in the more frankly invasive regions of the primary tumor. These findings are consistent with *TP53* mutations occurring later than *KRAS* mutations in CRC tumorigenesis as proposed by Fearon and Vogelstein, although the vast majority of *TP53* detected in metastases are also present in the matched primary tumors.

In an important study using both Sanger sequencing as well as a BEAMing assay in a subset of cases, Jones *et al.* assessed the status of 233 somatic mutations in paired samples of primary tumors and metastatic lesions from ten patients. They found that 226/233 (97%) of these genetic events were shared among all sites of disease (30) and concluded that virtually all of the mutations necessary for metastasis are already present in all of the cells of the antecedent carcinoma. Only seven mutations in *PLCG2*, *CORO1B*, *KCNC4*, *CRB13*, *ENPP2*, *GPR50* and *P2RY14* were detected exclusively in metastases, but their clinical relevance was not further investigated. In that study, the authors used mathematical modeling to gain insights into the relative timing of the birth of founder cells that gave rise to various tumor cell populations. Based on their analysis, they suggested that the average interval between the birth of a deeply invasive primary carcinoma founder cell and the liver metastasis founder cell is less than 2 years.

Massively parallel sequencing studies

In recent years, next-generation sequencing (NGS) technologies have revolutionized many aspects of cancer biology by allowing researchers to characterize the molecular landscape of different tumor types in unprecedented detail. They have also provided substantial insights into intratumor heterogeneity and tumor evolution by enabling whole exome sequencing of multiple tumors from the same patient. In the field of CRC, the use of NGS to study clonal evolution has been limited, and the few studies performed to date have produced somewhat conflicting results.

In one of the first studies, Vermaat *et al.* compared the genomic profiles of 21 pairs of primary tumors and subsequently resected liver metastases (31). Ten patients were chemo-naïve, while the remaining eleven patients received treatment between removal of their primary tumor and metastasectomy. DNA from FFPE archived material was analyzed using a targeted, PCR-based deep-sequencing assay that included all exons of 1,264 genes with a mean and median coverage of 156X and 85X, respectively. They reported that when all variations were considered, there were dissimilarities in the *KRAS* and *EGFR* mutational status between matched samples in 52% and 86% of patients, respectively, while modest variability was observed for *HRAS*, *PIK3CA*, *FLT1*, *NRAS* and *BRAF*. The nature of these observed variations was not clear and in fact, none of the *EGFR* mutations were predicted to result in a protein change. No significant differences were found between the chemo-naïve and the treated group. Based on their results, the authors concluded that there are substantial genetic differences between primary tumors and metastases and that metastases may provide a better "predictive window" for targeted therapy than the primary cancer. However, this conclusion would not be fully supported by the data if the alterations that accounted for the apparent heterogeneity in this study were not clinically relevant.

In a subsequent analysis using whole exome sequencing as well as high resolution copy number variation, Lee *et al.* compared the genomic profile of 15 primary CRC and matched liver metastases (32). They reported that eight cases (53%) shared mutations in key CRC-related genes such as *APC*, *KRAS*, *TP53*, *SMAD4*, *BRAF* and *FAT4* and were also closely related based on analysis of their copy number abnormalities. By contrast, in seven cases (47%) the primary tumor and metastasis were genetically different, as they did not share any mutations in the CRC-related genes and also did not cluster together after unsupervised hierarchical clustering of copy number changes. The authors proposed that in approximately half of CRC, the metastasis originates from a group of genetically distinct clones within the primary tumor, demonstrating a polyclonal model of disease progression, and recommended the evaluation of metastatic sites in the context of treatment decision-making. In that study, the authors reported that in four cases the liver metastasis was hypermutated and showed

deficiency in mismatch repair proteins, while the primary tumor was microsatellite stable and not hypermutated. Such a discrepancy would be unusual in CRC and, in general, testing of either a primary tumor or a metastasis is acceptable for evaluation of microsatellite instability. Moreover, the hypermutated cases showed a high-degree of copy number variation, a finding that conflicts with many prior studies including The Cancer Genome Atlas (TCGA) Network study where hypermutated tumors were reported to have far fewer copy number aberrations than non-hypermutated tumors (19). These latter findings raise concerns about the overall validity of the conclusions in this study.

A whole genome sequencing study of two cases found the majority of the mutations were shared between the primary tumor and the metastatic sites (33). This was especially true for key CRC-related mutations in *APC*, *KRAS* and *TP53*. The two cases differed in terms of their evolution pattern. In one case the metastasis shared >95% of the alterations found in the primary tumor, suggesting dissemination after full establishment of the founding primary clone. In the other case, approximately 25% of the mutations found in the primary tumor were not present in the metastasis, suggesting an earlier dissemination of the metastatic clone followed by the acquisition of additional mutations at both sites. Metastasis-specific mutations predicted to be deleterious (*ESR1*, *FBXW7*, *DCLK1*, *PHF6*, and *FAT2*) were also reported in this study; however, their role in disease progression remains to be determined.

Our group performed targeted sequencing of 230 cancer-associated genes in 69 microsatellite stable matched pairs. All cases underwent fingerprinting to ensure that matched pairs were from the same patient, and the mean target coverage was 692X (34). None of the patients had received anti-EGFR therapy though some had received chemotherapy. We found that 79% of all somatic alterations were shared between the primary tumor and the metastasis. When considering only the genes that were significantly mutated in the non-hypermutated CRC cases in the TCGA study, the concordance rate rose to 93%. Rare discordant events were observed in the *APC* gene and no discordant events in the *KRAS*, *NRAS* or *BRAF* genes. These findings confirm that genetic alterations that occur early in colorectal carcinogenesis persist through tumor evolution and show an exceedingly high level of concordance between the primary tumor and metastasis. Differences were found in a small number of cases in events observed in the *TP53*, *SMAD4* and *PIK3CA* genes. In the *PIK3CA* gene, we observed discordant

results in 4/15 somatic events including hotspot mutations E542K and E545K. Private mutations of unknown significance were also found in the *PIK3CD*, *PIK3CG*, *PIK3C2G*, *PIK3R1* and *PTEN* genes. These findings suggest that intratumor heterogeneity might be increased in the PI3K pathway in CRC, an observation that was also made in a more recent study by a different group (35). Parallel evolution convergent on the same gene was observed in two cases in our study: in one case, the primary tumor and metastasis showed different *TP53* mutations, while in another case there were different hotspot mutations in *PIK3CA*.

We did not observe a significant difference in the degree of mutational concordance between those cases where primary tumors and metastases were resected at the same time compared to those where there was a time interval between the two resections. Among patients whose tumors were concurrently resected, those that did not receive prior treatment were more likely to harbor discordant mutations compared to those that received prior therapy. Additionally, pre-treated patients with concurrently resected tumors were less likely to harbor primary-only mutations than patients with subsequent resections where only the metastasis received treatment. These findings raise the possibility that prior treatment results in a decrease of apparent intratumor heterogeneity, though larger studies are needed to further explore this hypothesis.

Conclusions

Studies to date have compared the genomic profile of primary CRC tumors to matched metastases using CGH as well as various sequencing methods, including most recently massively parallel sequencing techniques. Most of the genotyped metastases to date have been from the liver as they are frequently resected with a curative intent. These studies have established the clonal relationship of primary tumors and metastases by showing that in the vast majority of cases, matched pairs share a significant number of genetic events. Clonally unrelated tumors may be encountered in those patients harboring more than one primary tumor, and caution should be used in clinical practice when such patients undergo molecular testing.

Clonally related primary tumors and metastases are not identical; and both primary-private and metastasis-private genetic events are often present, consistent with a branched evolution pattern. The frequency of clonal divergence appears to vary among different types of genetic events.

Alterations that occur early in CRC pathogenesis, such as mutations in *APC* and *KRAS*, persist through tumor evolution and show an exceedingly high concordance between primary tumors and metastases. This would suggest that molecular testing of the primary tumor to determine eligibility for anti-EGFR therapy is appropriate in most clinical scenarios. Patients who have already received anti-EGFR therapy constitute an important exception as anti-EGFR therapy can promote selection of RAS mutant subclones in tumors that are otherwise *KRAS* wild-type (36,37). It should also be noted that given the low overall frequency of *BRAF* and *NRAS* mutations in CRC compared to *KRAS*, the number of *BRAF* and *NRAS* mutant tumors genotyped to date with the goal of examining patterns of tumor evolution remains very limited.

Events in other significantly mutated genes such as *PIK3CA*, *TP53* and *SMAD4* also tend to be identical between primary tumor and metastasis, although the level of clonal divergence seems to be higher compared to *KRAS*. In these genes, we have also observed evidence for convergent/parallel evolution where the primary tumor and metastasis show different functional aberrations in the same gene. In cases where one lesion is mutated while the other is wild-type, functional mutations have been observed both as primary-private events and as metastasis-private events. This finding is somewhat surprising and implies that in rare cases, functional events in established oncogenes or tumor suppressor genes may not be selected for in the metastatic clone or they may occur after dissemination of the cancer cells seeding the metastasis.

A higher level of intratumor heterogeneity is seen in genes that are not as frequently mutated in CRC. Many of these events do not appear to be functional *in silico*, suggesting that they may be passenger mutations and not clinically relevant. However, some alterations are predicted to be functionally deleterious and their effects need to be studied *in vitro* and *in vivo* to gain a better understanding of their role in tumor biology. Although a search for recurrent genetic aberrations that might mediate metastasis has not identified any specific mutations or focal copy number gains/losses, such events may be present at low frequencies and show high intertumor heterogeneity. Larger genomic studies coupled with functional experiments would be required to further investigate this hypothesis.

Additional studies are also needed to better define the extent of genetic diversity between different types of metastases, to explore the effect of treatment on tumor subclonal composition and to obtain more insight into

the relevance of intratumor heterogeneity in predicting therapeutic response and outcome. Such studies are difficult to perform currently as the only tissue available in patients with metastatic disease is often a small biopsy. This limitation can be overcome by harvesting and sequencing of tissue postmortem in patients with advanced disease as has been shown in the case of pancreatic cancer (38). Multi-region and longitudinal tumor sampling and sequencing through the course of the disease can also provide a more comprehensive profiling of heterogeneity and a better understanding of its impact on outcome. These studies are expensive and complex from a technical and regulatory aspect, but will be important in characterizing the landscape of subclonal diversity and its clinical relevance in different tumor types including CRC.

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Footnote

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