MOLECULAR ONCOLOGY 8 (2014) 129–141

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An integrative framework identifies alternative splicing events in colorectal cancer development

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ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form
17 September 2013
Accepted 8 October 2013
Available online 19 October 2013

Keywords:
Colorectal cancer
Alternative splicing
Exon arrays

ABSTRACT

Alternative splicing (AS) is a common mechanism which creates diverse RNA isoforms from a single gene, potentially increasing protein variety. Growing evidence suggests that this mechanism is closely related to cancer progression. In this study, whole transcriptome analysis was performed with GeneChip Human exon 1.0 ST Array from 80 samples comprising 23 normal colon mucosa, 30 primary colorectal cancer and 27 liver metastatic specimens from 46 patients, to identify AS events in colorectal cancer progression. Differentially expressed genes and exons were estimated and AS events were reconstructed by combining exon-level analyses with AltAnalyze algorithms and transcript-level estimations (MMBGX probabilistic method). The number of AS genes in the transition from normal colon mucosa to primary tumor was the most abundant, but fell considerably in the next transition to liver metastasis. 206 genes with probable AS events in colon cancer development and progression were identified, that are involved in processes and pathways relevant to tumor biology, as cell–cell and cell-matrix interactions. Several AS events in VCL, CALD1, B3GNT6 and CTHRC1 genes, differentially expressed during tumor development were validated, at RNA and at protein level. Taken together, these results demonstrate that cancer-specific AS is common in early phases of colorectal cancer natural history.

1. Introduction

Colorectal cancer (CRC) development is a model of cancerogenesis with a complex multistep process, involving accumulation of a significant number of genetic alterations of genes regulating key cellular processes (Fearon and Vogelstein, 1990; Sheffer et al., 2009). About 20–25% of colorectal cancer patients present with distant metastatic disease

Abbreviations: ncRNAs, non-coding RNAs; N, normal colon mucosa; T, primary colorectal cancer; M, liver metastasis; ASEs, alternatively spliced exons.

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http://dx.doi.org/10.1016/j.molonc.2013.10.004

(mainly in the liver) at diagnosis, and unresectable liver metastasis is associated with short survival (Gleisner et al., 2008; Jemal et al., 2005; Ogino and Goel, 2008).

Several studies have evaluated gene expression and genomic profiling of CRC (Cardoso et al., 2007; Habermann et al., 2008), focusing mainly on differential gene expression for disease phenotype classification (e.g. neoplastic *vs* normal tissue), but also on gene expression variability according to anatomical regions in normal colon (Birkenkamp-Demtroder et al., 2005; LaPointe et al., 2008). A recent comprehensive study involved exome sequence, DNA copy number, promoter methylation, mRNA and microRNA expression. As well as identifying recurrent mutated genes and methylation patterns, this study identified recurrent alterations in several pathways (e.g., WNT, PI3K, TGF-β, MAPK, p53) and found molecular signatures associated with tumor aggressiveness (Cancer Genome Atlas (2012)), thus reinforcing the concept that multiple genetic events are required to unleash the malignant progression of CRC and also that only a genome-wide approach can interpret complex scenarios in the biology of tumors.

Ample evidence now shows that most human genes undergo alternative splicing (AS) to express many transcript isoforms. The resulting protein isoforms play distinct roles, which contribute to increase the functional diversity of cells while maintaining a limited number of genes encoded by the genome.

Although splicing events do not always have functional consequences, it is clear that this process has great potential to produce a significant biological effect in several cell processes. For example, there are now several studies demonstrating that splicing alterations occur in many cancers (Pal et al., 2012). A quantitative estimate of splicing disruption in cancer has also been attempted, and the expression of normal splice variants was found to be widely and significantly disrupted in at least half the cancers studied (Ritchie et al., 2008). Until now, a relatively small number of studies have addressed the role of AS in tumors from breast, brain, lung and colorectal cancer, from which it appears that splicing alterations are quite common in cancer (Germann et al., 2012). In this regard, alternatively spliced proteins are particularly important in oncology, since they may contribute to the etiology of cancer, be involved in the metastatic process (Gutschner et al., 2013), serve as prognostic biomarkers (Brinkman, 2004) and provide tumor selective drug targets.

In addition, transcriptome complexity has gradually become appreciated in the last few years. Several classes of non-coding RNAs (ncRNAs) control expression at multiple levels, acting as epigenetic (Taft et al., 2010), transcriptional (Zardo et al., 2012) and post-transcriptional regulators (Bisognin et al., 2012; Lionetti et al., 2009) in normal development, physiology and, when dysfunctional, disease conditions.

Control of RNA processing is currently recognized as an essential component of gene expression regulation. For instance, alternative cleavage and polyadenylation play important roles in CRC development (Morris et al., 2012). RNA-based processes are definitely involved, either as causative entities, modulating influences, or as compensatory responses to disease (Ward and Cooper, 2010).

More than 95% of human genes encode splice isoforms, some of which exert antagonistic functions (Miura et al., 2012; Pan et al., 2008; Wang et al., 2008). Alternative splicing (AS) increases the diversity of both ncRNAs and coding transcripts, reflecting protein isoforms and directly influencing protein–protein interaction networks (Ellis et al., 2012). AS is accurately controlled, both spatially and temporally, by the interplay of cis-acting signals of trans-acting elements (Kornblihtt et al., 2013; Miura et al., 2011). The latter may comprise splicing machinery components as well as trans-acting ncRNAs with regulatory roles.

Over 50% of disease-causing mutations affect splicing (Tazi et al., 2009). Several splice variants are commonly found to be enriched in cancer tissue compared with normal surrounding tissue. Splicing changes may result from mutations within intronic or exonic splicing elements in cancer genes. However, aberrant splicing often involves transcripts from non-mutated genes, indicating defects in splicing effectors or regulators (Ward and Cooper, 2010). It should be noted that the metastasis-associated ncRNA MALAT-1 modulates AS, controlling the ratios among various isoforms through its interaction with the serine/arginine-rich (SR) family of nuclear phosphoproteins of the splicing machinery (Tripathi et al., 2010). Other advances in understanding molecular mechanisms and pathways modulating the AS of transcripts encoding key cancer proteins (caspase 9 AS regulation by phosphoinositide 3-kinase/Akt pathway (Goehe et al., 2010); SLC39A14 CRC regulation by the Wnt pathway (Thorsen et al., 2011)) have emphasized the importance of AS deregulation in all aspects of cancer aetiology. Aberrant splicing in cancer may increase the oncogenic potential of protein variants and promote cancer progression (Germann et al., 2012). Specific splicing events products may be signatures identifying cancer subtypes, predicting clinical outcomes or indicating treatment choices. It was the knowledge that the AS of Bcl-2 generates both pro- and anti-apoptotic proteins, whose combination regulates the apoptosis machinery critical for cell fate (Akgul et al., 2004), which paved the way for the development of novel anticancer drugs affecting the AS of Bcl-x and other human apoptotic genes (Shkreta et al., 2008). Modulation of splicing through small and anti-sense RNA molecules is also a powerful approach against disease causes and effects.

In this view, identification of cancer-associated splicing events and differential isoform expression between cancerous and normal tissues is a key issue in ongoing cancer research.

A few studies have investigated AS in colon cancer by means of exon arrays. Early in this field, Gardina et al. examined 20 paired tumor-normal colon cancer samples from 10 patients, predicting and partially validating specific splicing events mostly affecting cytoskeletal, extracellular and cell–cell interaction proteins (Gardina et al., 2006). Thorsen et al. studied tissue- and tumor-specific AS in various types of tumors, and validated six out of 23 detected colon cancer-specific AS events, only two of which were not reported by Gardina et al. (Thorsen et al., 2008). Mojica and Hawthorn reported exon array-based data from 13 non-neoplastic colonic epithelial cells from 10 patients, and directly compared them with previously mentioned matched samples, showing the complexity of AS and highlighting the limitations of current transcript annotations (Mojica and Hawthorn, 2010). More