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RESEARCH ARTICLE

Comprehensive analysis of *ALK*, *ROS1* and *RET* rearrangements in locally advanced rectal cancer

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Abstract. Gene rearrangements, such as anaplastic lymphoma kinase (*ALK*), c-ros oncogene 1 receptor tyrosine kinase (*ROS1*), rearranged during transfection (*RET*) and neurotrophic receptor tyrosine kinase 1 (*NTRK1*), identified in cancer have been indicated to be robust therapeutic targets in lung carcinomas. However, a few studies have focussed on locally advanced rectal cancer (LARC). The discovery of novel gene fusions is also valuable for LARC research. We used mass spectrometry-based assays and RNA sequencing to detect both known *ALK*, *ROS1*, *RET* and *NTRK1* rearrangements and novel gene fusions in LARC patients. FusionMap was also used to find gene fusions. None of the *ALK*, *ROS1*, *RET* or *NTRK1* gene fusions were detected by mass spectrometry-based assays or RNA sequencing. Three fusion candidates, integrin subunit beta 7 (*ITGB7*)-*ROS1*, lamin A/C (*LMNA*)-*NTRK1* and Golgi-associated PDZ and coiled-coil motif containing (*GOPC*)-keratin 8 (*KRT8*), showed relatively high junction-spanning reads by the FusionMap algorithm, but did not pass validation. These results suggest that no *ALK*, *ROS1* or *RET* rearrangements were found in LARC.

Keywords. rectal neoplasms; neoadjuvant therapy; RNA sequencing; mass spectrometry.

Introduction

Fusion genes form as a result of chromosomal rearrangements from abnormal transcription and have been shown to act as drivers of malignant transformation and progression in many cancers (Annala *et al.* 2013). For example, anaplastic lymphoma kinase (*ALK*), c-ros oncogene 1 (*ROS1*), ret proto-oncogene (*RET*) and neurotrophic receptor tyrosine kinase 1 (*NTRK1*) can activate kinases, and a number of them have been found to be an important class of oncogenes associated with tumours (Stransky *et al.* 2014). Many of the recurrent gene fusions identified in cancer have been proven to be robust therapeutic targets and represent the driving force of modern precision therapeutics. For example, imatinib induces remission in leukaemia patients who are positive for *BCR–ABL1* fusions (Goldman and Melo 2003). Recently, crizotinib and ceritinib have produced significant clinical benefits in patients with lung carcinomas and mesenchymal tumours harbouring gene fusions (Luo *et al.* 2010; Sartore-Bianchi *et al.* 2016).

There is also growing evidence in support of important role of gene fusions in colorectal cancer (CRC) development and their potential therapeutic value. Recently, a novel *CAD–ALK* gene rearrangement was identified from one of the 487 CRC cases, and the treatment of this patient with the *ALK* inhibitor entrectinib resulted in a durable objective tumour response after the failure of previous standard therapies (Amatu *et al.* 2015). Le Rolle *et al.* (2015) discovered two novel *RET* gene fusions by targeted sequencing in colorectal adenocarcinoma and showed a response to treatment with *RET* kinase inhibitors including one primary rectal cancer. Zhou *et al.* (2018) reported a case of a primary undifferentiated pleomorphic sarcoma of the lumbosacral region harbouring an *LMNA–NTRK1* gene fusion in a patient who failed to respond to treatment with neoadjuvant

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radiotherapy, extensive resection and adjuvant chemotherapy but had a durable clinical response to crizotinib. Further, the treatment of patients with NTRK fusion-positive cancers with a first-generation TRK inhibitor, such as larotrectinib or entrectinib is associated with high response rates (>75%), regardless of tumour histology (Cocco *et al.* 2018). Locally advanced rectal cancer (LARC) is the most common type of CRC but has a low pathologic complete response (pCR) rate with standard neoadjuvant chemoradiotherapy (nCRT). It is still a cumbersome problem in the clinic. To our knowledge, a very few studies have analysed the gene fusion profiles in LARC. The detection of *ALK*, *ROS1*, *RET* and *NTRK1* rearrangements, whether known or novel gene fusions in LARC is considered increasingly important for the development of new targeted therapies.

In this study, we focussed on four most important therapeutic targets *ALK*, *RET*, *ROS1* and *NTRK1* in LARC samples, which are reported in other cancers. Three high-throughput transcription analyses were performed, including targeted RNA sequencing run on the Ion Torrent sequencer, mass spectrometry-based assay, and targeted RNA sequencing data analysis by FusionMap. The purpose of this study was to explore whether these key fusion genes as well as novel fusions are also present in LARC to provide useful clues in precision medical research.

Material and methods

Patient cohort

This study is a single-centre, retrospective, clinical cohort study where 30 patients with locally advanced, confirmed carcinoma of the rectum as the first diagnosis, who did not receive any treatment were enrolled. The exclusion criteria were as follows: prior history of chemotherapy, immunotherapy, or radiation therapy; and unsuitability for CRT. The research was approved by the Ethics Committee of Sun Yat-sen University Cancer Center, and informed consent was obtained from all patients prior to collecting the specimens. Fresh enteric needle biopsy specimens (2 mm*2 mm*2 mm) were collected from the patients before receiving therapy, and the samples were stored at −80°C.

RNA extraction

Total RNA was extracted from fresh frozen tissues using the QIAamp RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and the concentration was measured using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32852). The Agilent 2100 Bioanalyzer was used to quantify and evaluate RNA integrity.

RNA sequencing

For each sample, 50 ng of RNA was reverse transcribed and amplified using the Oncomine Solid Tumor Library Assay (Thermo Fisher Scientific), and the protocol used was described in the Oncomine Solid Tumor Library Prep_RUO_UG user guide MAN0014028 Revision A.0. Libraries were ligated with barcoded sequencing adapters and then purified with Agencourt AMPure XP beads (Beckman Coulter) washed with 70% ethanol, and eluted in 50 μL of Low TE buffer. The libraries were quantified and sequenced on an Ion 318 v2 chip (Thermo Fisher Scientific) using an Ion Torrent PGM instrument (Thermo Fisher Scientific). Eight samples were batched on a single 318 PGM chip to give >150,000 reads per sample. Raw data were transferred to Torrent Suite software for alignment and to Ion Reporter software v4.4 for fusion calling. Fusion genes were detected when the sequence of one gene was found relocated to the breakpoint.

Mass spectrometry

Fifty nanograms of total RNA was converted to cDNA using the Invitrogen SuperScript VILO cDNA Synthesis kit (Life Technologies, Carlsbad, USA) according to the manufacturer's recommendations. cDNA concentration was measured using a Qubit fluorometer (Life Technologies, Grand Island, USA). In brief, 15–30 ng of cDNA per sample was amplified in the presence of a known copy number competitive template standard within a single multiplex reaction with primers designed immediately 3′ of the promoter and the kinase domain. The reaction was conditioned with ion exchange resin, and 5–15 nL was spotted onto a 384-pad Spectro-CHIP (Agena Bioscience, San Diego, USA). The masses were obtained by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy on a MassARRAY Analyzer 4 (Agena Bioscience, San Diego, USA).

An algorithm called FusionMap was used to detect gene fusions based on the raw data produced by the RNA sequencing assay described above. Fusion transcript breakpoints were validated by reverse-transcription PCR (RT-PCR) using 50 ng of RNA with Prime Script RT reagent. The primers were designed to span the fusion breakpoints detected by FusionMap using Primer3 software. Fragments containing the fusion breakpoint sequences were also synthesized as PCR controls. The PCR products were detected by electrophoresis using a 2% agarose gel. One-way ANOVA was used for statistical comparisons of patient characteristics, as shown in table 1.

Results

Patient cohort

Thirty patients with LARC were recruited from Sun Yat-sen University Cancer Center from January 2014 to January

2016. The clinical characteristics data are available in table 1.

RNA sequencing

The assay run on the Ion Torrent sequencer (Thermo Fisher Scientific) was used for the targeted sequencing of known and novel fusion junctions for *ALK*, *RET*, *ROS1* and *NTRK1*. The total mapped reads of the 30 samples ranged from 190580 to 1552225 with a mean of 564535, which indicated that all of the samples passed the quality control (total mapped reads >20000). Five housekeeping genes, which were used for quality control to ensure that the RNA integrity was reliable, were all detected in the 30 samples. Negative results of the gene fusions were reported overall among the 30 samples (table 2).

Mass spectrometry-based assay

The mass spectrometry-based assay based on the MassARRAY platform was used for the detection of *ALK*, *ROS1* and *RET* rearrangements. The software makes translocation calls based on the relative 3′ and 5′ expression of each gene (*ALK*, *ROS1* and *RET*). Unincorporated dNTPs were inactivated, and then a single base extension reaction was performed to generate a unique mass product for each site. Translocation will be reported when a gene's Z value is >3. Further, internal quality control markers were also employed to ensure reliable results by assessing sample yield and integrity. All 30 samples passed the quality control. None of the 30 samples reported translocations for *ALK*, *ROS1* or *RET*, while the positive controls detected translocation (figure 1).

FusionMap data analysis

For further validation, single-end reads were generated from the raw sequence data of the 30 rectal cancer samples. A total of 3928 fusion transcripts with junction seed counts >3 were obtained. Among them, the most common form of transcripts was the five housekeeping genes. There were three fusion candidates that showed relatively high junction-spanning reads (table 3). Unfortunately, the RT-PCR results were all negative (figure 2). Therefore, they may be artifacts of the sequencing/bioinformatic mapping process, particularly considering that they did not pass validation.

Table 1. Clinical characteristics of 30 rectal cancer patients.

	No. of patients	Percentage	*P* value
Sex			0.119
Male	23	77	
Female	7	23	
Age (year)			0.947
Median (range)	61 (43–73)		
uT stage			0.721
II	2	7	
III	18	60	
IV	9	30	
X	1	3	
uN stage			0.294
0 (≥12 lymph nodes examined)	2	7	
+ (>1 lymph nodes positive)	27	90	
X (<12 lymph nodes examined or N/A)	1	3	
Tumour regression grade (TRG)[†]			
1	2	7	
2	6	20	
3	17	57	
4	1	3	
Unknown	4	13	

[†]Pathologic tumour regression grades after preoperative chemoradiation therapy as described by Mandard *et al.* (1994).

Table 2. Detection results of targeted RNA sequencing.

Gene	Positive for fusion reads	Positive for 3′/5′ read ratio	Total positive
ALK	0/30	0/30	0/30
ROS1	0/30	0/30	0/30
RET	0/30	0/30	0/30
NTRK1	0/30	0/30	0/30

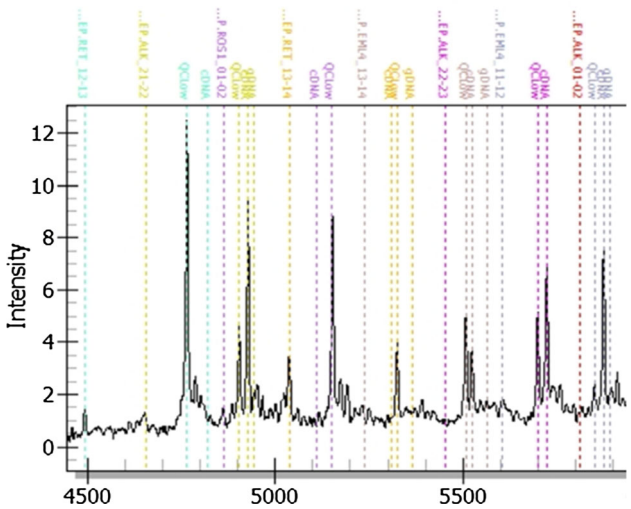

Figure 1. Detection of *ALK*, *ROS1* and *RET* gene fusions in the ALK–EML4 positive control sample. When a fusion is presented, the characteristic mass spectrometry peak of cDNA will appear higher than that of gDNA and the QC low in the analytical output of the MassARRAY system.

Table 3. Detection results of FusionMap suitable for validation.

Sample ID	Unique cutting position count	Seed count	Gene 1	Gene 2	Fusion junction sequence	Fusion gene
13	15	428	*ITGB7*	*ROS1*	…GTGAGACCCCActccaccctctattagtgcacctca	*ITGB7->ROS1*
2	4	16	*NTRK1*	*LMNA*	…AGTTCCTCCTTCggtgggcaggcacgtggccgct	*NTRK1->LMNA*
16	4	32	*GOPC*	*KRT8*	…AATATCCTCGTActagtctcgccccatatacttcag	*GOPC->KRT8*

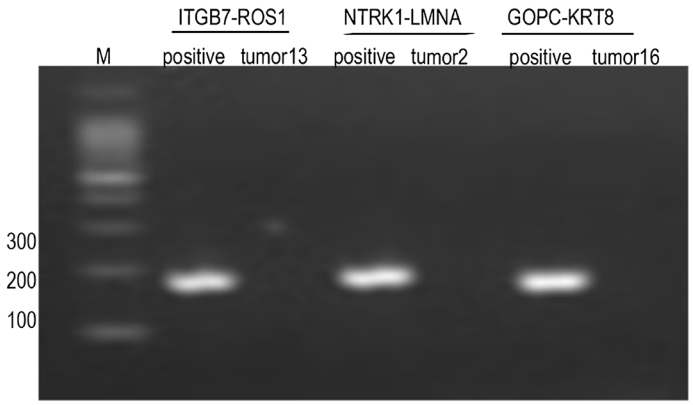

Figure 2. Characterization of *ITGB7–ROS1*, *LMNA–NTRK1* and *GOPC–KRT8* transcripts by RT-PCR. The positive control lines, which contained the fusion junction fragments, showed the expression of *ITGB7–ROS1*, *NTRK1–LMNA*, or *GOPC–KRT8*. No bands were detected on the right side for samples 13, 2 and 16.

Discussion

Over the past decade, the discovery of druggable genetic alterations in *ALK*, *ROS1* and *RET* gene fusions has led to many advances in NSCLC (Davies *et al.* 2012; Lipson *et al.* 2012; Vaishnavi *et al.* 2013; Farago and Azzoli 2017). *ROS1* fusions can lead to constitutive kinase activity and sensitivity to *ROS1* inhibitors, such as crizotinib, in lung cancer (Shiroguchi *et al.* 2012). *RET* fusions are potential therapeutic targets of existing multitargeted kinase inhibitors, such as cabozantinib, in lung cancer (Suehara *et al.* 2012; Takeuchi *et al.* 2012). These genes have also been shown to be robust therapeutic targets in CRC. Lee *et al.* (2015) recently reported two of 74 colorectal adenocarcinoma cancer cases, including one of 25 rectal adenocarcinoma cases, with the *NTRK1* rearrangement. Sartore-Bianchi *et al.* (2016) found that *TPM3–NTRK1* gene fusions are a recurring event in CRC (three out of 147, 2%) and are a clinically relevant target for therapeutic intervention (Park *et al.* 2016). Fusion genes in CRC research are conducive to the clinically individualized use of drugs to improve quality of life, as well as the identification of new target sites to promote the development of targeted drugs. The value of those key gene fusions in LARC is still not confirmed, while more information is needed for precise nCRT decision-making. The identification of fusion genes is expected to be a supplemental method for LARC, as it has contributed to lung cancer, especially patients who failed nCRT treatment or those with refractory LARC. Approximately, there is only 15–20% pathologic complete response (pCR) after nCRT (Julien and Thorson 2010). The potential role of fusion genes in therapy gives new hope to the patient with treatment failure after nCRT. At the early stage, fusion gene research in LARC has seldom been reported. Encouragingly, some gene fusions have been found in patients with rectal cancer and were validated to be involved in the response to kinase inhibitors (Le Rolle *et al.* 2015; Lee *et al.* 2015). We used high-throughput transcription analyses to detect *ALK*, *ROS1*, *RET* and *NTRK1* gene fusions in LARC samples as an exploratory study.

The recent development of high-throughput transcriptome analysis provides a powerful approach to profile gene fusions (Cabillic *et al.* 2014; Choi *et al.* 2018). For example, spectrometry-based assays and targeted RNA sequencing are not only able to detect multiplexing gene fusions but also able to discover novel gene fusions (Ozsolak and Milos 2011; Sakai *et al.* 2012). Subsequently, these methods have shown similar analytical sensitivity and specificity as common diagnostic methods (Rogers *et al.* 2017). This finding confirms that spectrometry-based assays and targeted RNA sequencing are efficient methods in fusion gene identification and research. FusionMap is a useful and well-defined algorithm that can detect fusion events without prior knowledge of potential fusion regions in datasets from RNA-seq (Ge *et al.* 2011). In our study, no gene fusions for *ALK*, *ROS1*, *RET* or *NTRK1* were detected by the targeted

RNA sequencing run on the Ion Torrent sequencer or by the mass spectrometry-based assay. Although all the tests passed the QC, the positive controls presented positive results. When FusionMap was run, three fusion candidates, *ITGB7–ROS1*, *LMNA–NTRK1* and *GOPC–KRT8* showed active signs. *LMNA–NTRK1* was identified in CRC and was associated with sensitivity to entrectinib (Sartore-Bianchi *et al.* 2016). The other two fusion genes *ITGB7–ROS1* and *GOPC–KRT8* are novel fusion genes which have not been reported before. *ITGB7* is a new marker for minimal residual disease detection in acute lymphoblastic leukaemia (Coustan-Smith *et al.* 2011). *ITGB7–ROS1* may play a critical role in cell survival and be a target of the *ROS1* kinase crizotinib. The *GOPC–ROS1* rearrangement was reported as an acquired resistance mechanism to osimertinib and response to crizotinib combined treatments in lung adenocarcinoma (Zeng *et al.* 2018). *GOPC* and *KRT8* play a role in the prognosis of various tumours. The expression of *GOPC* was recently found to be a poor prognostic marker in colorectal cancer (Ohara *et al.* 2017). *KRT8* upregulation can promote tumour metastasis and is predictive of poor prognosis in clear cell renal cell carcinoma (Tan *et al.* 2017). Encouraged by all these reports, we speculate that the fusion candidates *ITGB7–ROS1*, *LMNA–NTRK1* and *GOPC–KRT8* detected by FusionMap may be related to tumour development in LARC. Unfortunately, the RT-PCR results showed that they were not true rearrangements.

The regretful negative results we found by all three methods may be mainly due to the lower fusion frequency of those targeted genes in LARC or tumour heterogeneity. As reported before, *RET* fusion kinase occurs nearly in one-third of papillary thyroid cancer and 1–2% of lung adenocarcinoma but only occurs at a 0.2% frequency in CRC (Le Rolle *et al.* 2015). The differences in experimental approaches and the low frequency of fusions may also lead to conflicting results regarding its prevalence and relevance. As Dr Kopetz presented at the Chinese Society of Clinical Oncology (CSCO) Annual Meeting (Xiamen, China, 19–23 September 2018), fusion genes in colorectal cancer are rare but actionable. The small sample size may have also influenced the regretful negative results. The addition of more LARC samples and the use of pertinent sample types, such as adenocarcinoma only, T4 stage only, one population only or samples gathered after surgery may increase the detection rate for gene fusions. The detection of other gene fusions besides *ALK*, *RET*, *ROS1* and *NTRK1* may also be a meaningful choice in LARC research.

In conclusion, the advanced multiplexing high-throughput transcription analysis technologies were used to detect *ALK*, *ROS1*, *RET* and *NTRK1* gene fusions in LARC samples, and none of the confirmed fusion genes were found.

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