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Tumour suppressor properties of miR-15a and its regulatory effects on BCL2 and SOX2 proteins in colorectal carcinomas

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ABSTRACT

Objectives: In this study, we aimed to investigate the expression pattern, clinicopathological significance and tumour suppressive properties of miR-15a in patients with colorectal carcinomas.
Methods: Tissue samples from 87 patients with primary colorectal carcinomas, 50 matched metastatic lymph node and 37 non-neoplastic colon (control) were prospectively recruited. The expression level of miR-15a was measured by quantitative real-time polymerase chain reaction. Restoration/overexpression of the miR-15a was achieved by exogenous transfection. Four colon cancer cell lines (SW480, CaCO2, SW48 and HCT116) and a non-cancer colon cell line (FHC) were also used for examining the miR-15a induced tumour suppression properties using various in-vitro and immunological assays.
Results: Downregulation of miR-15a was noted in ~ 62% of the colorectal carcinoma tissues and it was positively correlated with the presence of cancer recurrence in patients with colorectal carcinomas ($p = 0.05$). Also, these patients with low miR-15a expression showed relatively shorter survival time when compared to those with miR-15a overexpression. Following miR-15a exogenous overexpression, colon cancer cells showed reduced cell proliferation, low colony formation, less cell invasion properties and mitochondrial respiration when compared to control cells. In addition, BCL2 and SOX2 proteins showed a significant downregulation following miR-15a overexpression suggesting its regulatory role in cancer growth, apoptosis and stemness.
Conclusion: This study has confirmed the tumour suppressor properties of miR-15a in colorectal cancers. Therefore, its modulation has potential implications in controlling various biological and pathogenic processes in colon carcinogenesis via targeting its downstream proteins such as BCL2 and SOX2.

1. Introduction

MicroRNA (miRNA)s play a key role in the regulation of gene expression via pairing with the complementary sequence in the 3′-untranslated region (UTR) of a target mRNA and thereby regulating their protein expression [1]. Recent studies have confirmed that miRNAs expressions are critical in various cellular functions such as cell differentiation, cell cycle progression, stress response and apoptosis [2]. It has been postulated that approximately 30% of the human genome is regulated by miRNA [3]. It is worth noting that miRNAs can function either as a tumour suppressor or as an oncogene and are frequently interchangeable in its expression patterns in human cancers [4–7]. MiR-15a is one of the first group of miRNAs identified exhibiting significant role in carcinogenesis and deletion of miR-15a was first reported in patients with chronic lymphocytic leukaemia [8]

MiR-15a act as a tumour suppressor in carcinogenesis via inhibiting cell proliferation and promoting apoptosis both in vitro and in vivo [9]. Also, miR-15a exhibits its tumour suppressor properties via targeting multiple oncogenes, including *BCL2*, *MCL1*, *CCND1*, and *WNT3A* [10,11]. Downregulation of miR-15a has recently been reported in patients with colorectal carcinoma and modified miR-15a has therapeutic potential for improving treatment of colorectal carcinomas in

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advanced stages via regulating its target proteins [12,13]. Furthermore, miR-15 downregulation correlates with poor patient prognosis and advanced pathological stages in patients with colorectal carcinoma [12]. However, there are no reports on the biological impacts of miR-15a expression in colon cancer cells. In addition, clinicopathological correlations with cellular changes in vitro have never been studied in patients with colorectal carcinoma. Thus, in this study, we aimed to analyse the expression profiles and clinicopathological significance of miR-15a a large cohort of patients with matched primary and metastatic colorectal tissues. Also, the cellular ramifications of miR-15a in colon cancer cells were investigated in-vitro.

2. Materials and methods

2.1. Selection of patients

The patients were selected for this study had surgical resection of colorectal adenocarcinomas between January 2006 and December 2013 in Queensland, Australia. Ethical approval for this study was obtained from the Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC). After histopathological analysis, tissue samples from 124 patients (37 non-neoplastic; 87 primary adenocarcinomas together with 50 matched lymph nodes with metastatic adenocarcinoma) were used for this study. The non-neoplastic tissues (control) used in this study were recruited from patients diagnosed with hyperplastic polyps, diverticular diseases and intestinal volvulus. We select one tissue sample from each surgically resected specimen for miRNA extraction.

2.2. Clinicopathological characteristics

The demographic data and macroscopic features: age, gender, size and location of the tumours were documented for each patient in the study. A pathologist reviewed all the pathological characteristics of the carcinomas (AKL). Only adenocarcinomas were included in the study. The colorectal adenocarcinomas were classified, graded, and according to the World Health Organisation (WHO) criteria [14]. The carcinomas (66 from colon and 21 from rectum) were staged according to TNM (a tumour, lymph node and metastases) classification adopted in American Joint Committee on Cancer (AJCC) [15]. Both conventional (n = 78) and mucinous (n = 9) adenocarcinomas (> 50% mucin distribution in conventional adenocarcinomas) were included in the study [16]. In addition, lymphovascular permeation were also recorded for all patients with colorectal adenocarcinoma. Microsatellite instability (MSI) was performed in 33 primary adenocarcinomas. The test was done by immunohistochemistry on four proteins (MLH1, PMS2, MSH2 and MSH6). Colorectal carcinoma with the absence of any of these protein markers was considered as MSI high cases.

The management of all the patients was discussed in the weekly multidisciplinary team meeting and followed up by the same team of clinicians. The survival rate of the patients was calculated from the date of surgical resection of the colorectal carcinomas to the date of death or last follow-up.

2.3. Cell culture

Four human colon cancer cell lines derived from different pathological stages (CaCo2, HCT116, SW480 and SW48) were used in this study and were purchased from American Type Culture Collection (ATCC). A non-neoplastic colon epithelial cell line, FHC (also obtained from ATCC) was used as a control. These cell lines were cultured and maintained according to the previously published protocol [6,7].

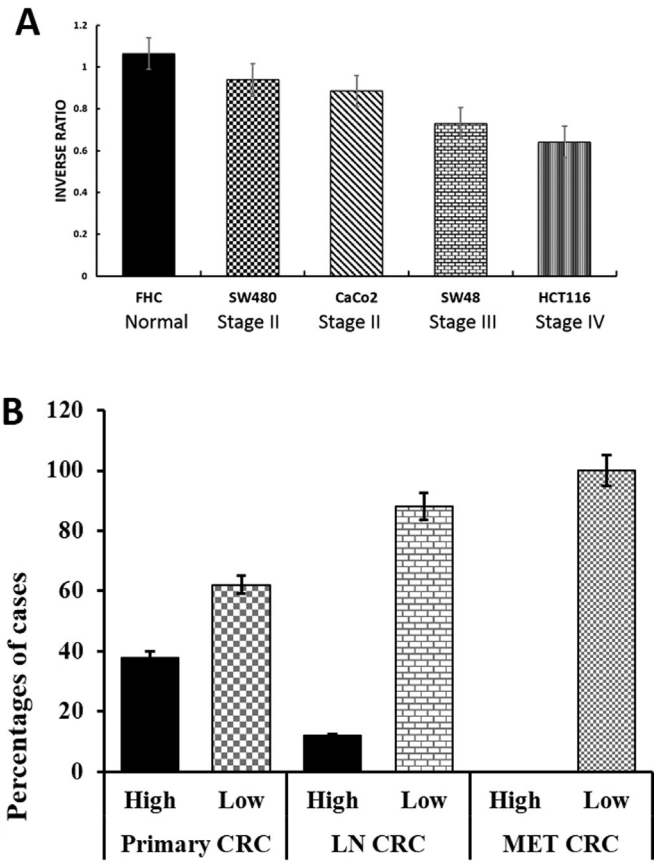

Fig. 1. Expression levels of miR-15a colorectal cancers. A- Colon cancer cells with advanced pathological stage, HCT116 (stage IV), SW48 (stage III), SW480, and CaCo2 (stage II) showed reduced expression of miR-15a compared to normal epithelial cell line (FHC). Inverse ratio of miR-15a versus RNU6B (control miRNA) (expression ratio) was used to illustrate the miRNA expression levels in various cell lines. B- Expression levels of miR-15a were altered in primary colorectal cancer (CRC) tissues, CRC lymph node tissues and distant metastasis tissues. Higher expression levels of miR-15a were noted in primary CRC tissues while lower miR-15a expression was found in lymph node and distant metastasis tissues.

2.4. miRNA extraction and quantitative real-time polymerase chain reaction (PCR)

The tissue samples were fixed in formalin and later embedded in paraffin for sectioning. Each tissue block was sliced into 7 µm and transferred into an Eppendorf tube for purification of miRNA. Isolation of miRNAs from both tissues and cell lines were performed as previously reported [6,7]. After purification, complementary DNA (cDNA)s from these miRNAs were prepared using a commercial reverse transcription kit, miScript II RT Kit (Qiagen, Hilden, Germany). The cDNAs were diluted using RNAase free water to 1.5 ng/µl for quantitative real-time PCR (qRT-PCR).

Each PCR reaction for miR-15a, RNU6B (internal control miRNA) was performed in triplicates and the mean cycle threshold (Ct) value of these PCR reactions was documented. Data analysis for determining miR-15a expression was performed using the $2^{-[delta][delta]Ct}$ (fold change) method [6,17]. Fold change greater than 2 was denoted as high miR-15a expression, while low miR-15a expression was regarded as a fold change of less than one [6,17]. The Ct ratio of RNU6B versus miR-15a [expression ratio] was used to represent the miRNA expression in relative to control samples.