Choosing the right cell line for rectal cancer research

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UP TO DATE NO EFFECTIVE METHOD EXISTS THAT PREDICTS RESPONSE TO PREOPERATIVE CHEMORADIATION (CRT) IN LOCALLY ADVANCED RECTAL CANCER (LARC). NEVERTHELESS, IDENTIFICATION OF PATIENTS WHO HAVE A HIGHER LIKELIHOOD OF RESPONDING TO PREOPERATIVE CRT COULD BE CRUCIAL IN DECREASING TREATMENT MORBIDITY AND AVOIDING EXPENSIVE AND TIME-CONSUMING TREATMENTS. USING THE Gng4, c-Myc, Pola1, AND Rrm1 SIGNATURE, WE WERE ABLE TO ESTABLISH A MODEL TO PREDICT RESPONSE TO CRT IN RECTAL CANCER WITH A SENSITIVITY OF 60% AND 100% SPECIFICITY. THE AIM OF THIS STUDY WAS TO CHARACTERIZE c-Myc STATUS IN DNA, RNA AND PROTEIN LEVELS IN 3 TUMORAL CELL LINES (SW480, SW620 AND SW837) TO ESTABLISH THE BEST CELL LINE MODEL AND, SUBSEQUENTLY, CARRY OUT GENOME SILENCING OF c-Myc BY MEANS OF RNA INTERFERENCE (iRNA). TO STUDY THE EXPRESSION LEVELS OF c-Myc, WE USED POLYMERASE CHAIN REACTION (PCR) AMPLIFICATIONS AND SEQUENCING; QUANTITATIVE REAL TIME PCR (qRT-PCR); AND WESTERN BLOT ANALYSIS IN EACH CELL LINE. SW480 AND SW620 SHOWED A VARIATION A › G IN EXON 2, WHICH CAUSED A SUBSTITUTION OF ASPARGINE TO SERINE, AND SW837 REVEALED A G › A TRANSITION IN THE SAME, WHICH CAUSED A MUTATION AT CODON 92. THE THREE CELL LINES EXPRESSED c-Myc mRNA. SW837 SHOWED A DECREASE OF c-Myc EXPRESSION LEVELS COMPARED WITH SW480, AND SW620. AT PROTEIN LEVEL, SW620 SHOWED THE HIGHEST EXPRESSION OF c-Myc. ACCORDING TO THE RESULTS OBTAINED, WE CAN PERFORM c-Myc GENE SILENCING EXPERIMENTS TO ANALYZE THE ROLE OF THIS BIOMARKER IN RESPONSE TO TREATMENT.

C-MYC, RECTAL TUMOR, RESPONSE, CELL LINES.

Introduction

To date, none of the identified signatures or molecular markers in LARC has been successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice. Moreover, there has been little agreement between signatures, with scarce overlap in the reported genes. According to our previous reports, the Gng4, c-Myc, Pola1, and Rrm1 mRNA expression levels were a significant prognostic factor for response to treatment in LARC patients ($p<0.05$). Using this gene set, we were able to establish a new model to predict response to pre-operative chemoradiation in rectal cancer with a sensitivity of 60% and 100% specificity. These findings could be clinically relevant and support the use of mRNA levels to identify patients who respond to therapy (1).

CRT is the standard therapy for patients with LARC (2). In our previous papers, the LARC patients were treated with capecitabine which is enzymatically converted to 5-fluorouracil (5-FU) in the tumor, where it inhibits DNA synthesis and slows growth of tumor tissue (1,2). Based on previous research, c-Myc plays an important role in growth control, cell cycle progression, and stimulation of the G1-S transition (3,4). Understanding of the function of c-Myc could increase our understanding of the biology of the responder LARC patients but also may provide a novel therapeutic molecular target for clinical practice. For this reason, we design a new project with cancer cell lines and iRNA. To choose the best experimental model, we characterized the c-Myc status (DNA, RNA, and protein) in three colorectal cancer cell lines (SW480, SW620, and SW837). The c-Myc protein was detected in the cell lines, indicating that they are a good model to suppress c-Myc expression.

Material and methods

Analysis of c-Myc status in human cell lines was carried out by DNA, RNA, and protein assays.

Samples

We obtained DNA and RNA using standard procedures from 3 colorectal tumoral cell lines (Table 1). These cell lines were acquired in American Type Culture Collection (ATCC). Each of the cell lines was successfully cryopreserved and thawed quickly. Cell lines grew in RPMI 1640 supplemented with 10% of fetal bovine serum, and absence of Mycoplasma contamination was tested periodically by PCR.

Of every cell line we took cellular's pellet, which we then used to obtain DNA, RNA and proteins.

Mutational analysis of c-Myc

We amplified genomic c-Myc DNA from cell lines using specific primers for each exon (Table 2). PCR was performed

in a 20 mL final volume reaction containing: 1µL of DNA (100 µM), 2 µL of Buffer, 12.2 µL of water, 2 µL of Magnesium, 0.4 µL of dNTPs (10 mM each), 1 µL of each primer, and 0.2 µL of Taq polymerase (thermo scientific) under the following cycling conditions: 40 cycles of 94° C for 30 s, 55° C for 30 s and 72° C for 30 s. The PCR products were then analyzed by automatic sequencing (Applied Biosystems 3130 and 3130xl Genetic Analyzers).

Quantitative real time PCR

We optimized a sensitive and specific quantitative real-time PCR assay for detection of the c-Myc. One microgram of RNA was used for reverse transcription with M-MLV RT (using random hexamers). PCR reactions contained 1 µL cDNA, 5 µL qPCR SyBr GreenMaster Mix, 2 uL water, 2 uL primers for each gen. PCR conditions were 30 s at 95° C, 15 s at 95° C and 1 min at 60° C for 40 cycles. All samples were measured in triplicate. We designed specific primers for c-Myc and Gapdh (Table 3).

For each experimental sample, the amount of the c-Myc and endogenous reference (Gapdh) was determined from the standard curves. These standard curves were composed of five points obtained from two-fold serial dilutions of cDNA drawn from a pool of tumoral cell lines. We considered only experiments in which the linear relationship between Ct (threshold cycle) and the log of the amount of standard curve for c-Myc and Gapdh were higher than 0.99 (correlation coefficient). The c-Myc amount was then divided by the amount of Gapdh to obtain a normalized value. Gapdh gene was used as an internal control for RNA quality reverse transcription and to correct the variations in the degree of RNA degradation. Before performing this study we confirmed that expression of Gapdh was steady in a series of tumors to demonstrate the suitability of this gene.

Western blot

For Western blot analyses, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, 25 mM MgCl2, and supplemented with a phosphatase inhibitor cocktail). Protein concentration was determined by the Bradford assay.

50 µg of protein lysate was resuspended in sample buffer, separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked in PBS-T containing 3% powdered milk for 60 min and incubated in anti-c-Myc antibody (Cell Signalling) and Actin (SantaCruz) overnight. Blots were probed with conjugated anti-rabbit and anti-mouse secondary antibody respectively (Dako).

	SW837 (CCL235)	SW620 (CCL 227)	SW480 (CCL228)
Description	Rectal adenocarcinoma (stage IV)	Rectal adenocarcinoma (Duke's type C)	Colorectal adenocarcinoma (Duke's type B)
Tissue	Human's rectal	Human's colon but it comes from a lymphatic metastasis	Human's colon
Morphology	Adhesive epithelial cell	Adhesive epithelial cell	Adhesive epithelial cell

▲ **Table 1**. *Description of tumoral cell lines.*

Name	Type	Sequence
c-Myc-1	**Primer Forward**	**5´-ATAATGCGAGGGTCTGGACG-3´**
	Primer Reverse	5´-AGCTAACGTTGAGGGGCATC-3´
c-Myc-2	Primer Forward	5´-CCGCTTCTCTGAAAGGCTCT-3´
	Primer Reverse	5´-CAGGTACAAGCTGGAGGTGG-3´
c-Myc-3	Primer Forward	5´-CTCGTCTCAGAGAAGCTGGC-3´
	Primer Reverse	5´-GCTGGTGCATTTTCGGTTGT-3´
c-Myc-4	Primer Forward	5´-ACAACCGAAAATGCACCAGC-3´
	Primer Reverse	5´-CCCAAAGTCCAATTTGAGGCA-3´
c-Myc-5	Primer Forward	5´-GTCTTGAGACTGAAAGATTTAGCCA-3´
	Primer Reverse	5´-GTTGGGAGGGGGAAGAAACGA-3´

▲ **Table 2**. *Specific primers for amplifying genomic c-Myc DNA.*

Name	Type	Sequence
c-Myc (quantitative)	Primer Forward	5´- CGTCCTCGGATTCTCTGCTC-3´
	Primer Reverse	5´- GCCTGCCTCTTTTCCACAGA-3´
GADPH (qPCR-housekeeping-control)	Primer Forward	5´-AAGCAGTTGGTGGTGCAGGATG-3´
	Primer Reverse	5´-CGTAGACAAAATGGTGAAGGTCGG-3´

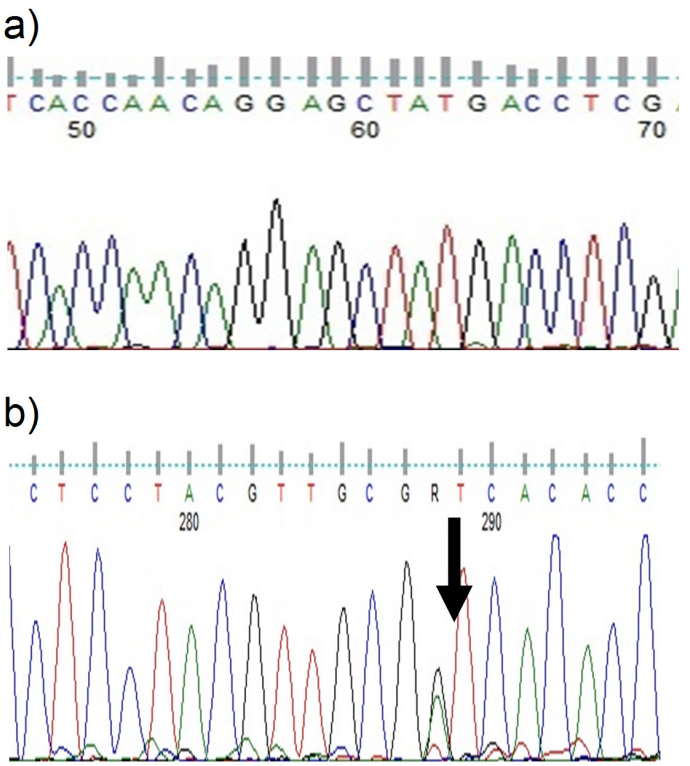
▲ **Table 3**. *Specific primers for qRT- PCR.*

Results

1. c-Myc genotype

The coding region of c-Myc was amplified from genomic DNA by PCR, as described previously. The cell lines, SW480 and SW620, showed a variation A > G in exon 2, which caused

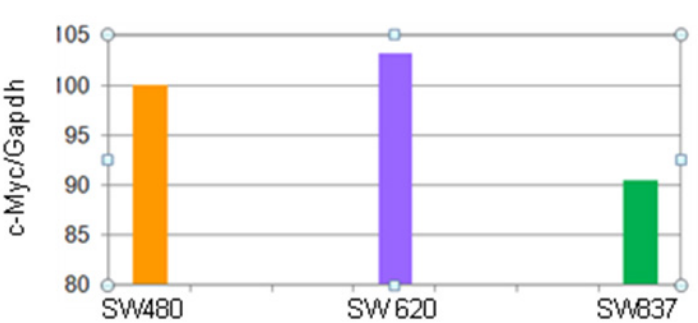
a substitution of aspargine to serine at codon (p.Asp258Ser) (Figure 1A). Direct sequencing of the SW837 cell line's PCR products revealed a G> A transition in exon 2, which caused a previously reported mutation at codon 92 (p.V92I) (Figure 1B) (5).



▲ **Figure 1**. *Genotyping of the c-Myc gene. a) At the top, the sequence of the genomic DNA of G>A transition (indicated by arrow) in SW480, and SW620; b) The G/A heterozygous variation is shown in the SW837.*

2. c-Myc expression levels in colorectal tumoral cell lines

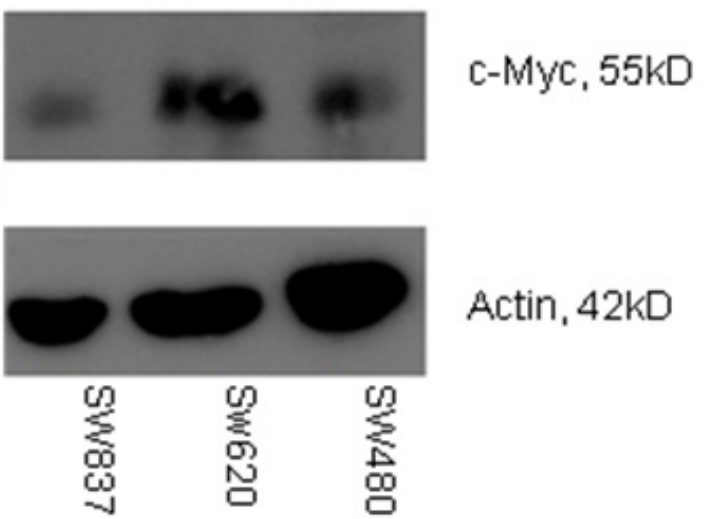
We determined the level of expression of c-Myc by qRT-PCR. RNAs from 3 cell line samples were extracted (Figure 2). The SW480, SW620 and SW837 cell lines expressed c-Myc mRNA. SW837 showed a decrease of c-Myc expression levels compared with SW480, and SW620. Furthermore, c-Myc expression in SW480 and SW620 was similar. Importantly, these two cell lines derived from the same patients, confirming that that there are genetic similarities between primary tumor and metastasis from the same patient.



▲ **Figure 2**. *c-Myc expression levels by qRT-PCR. The expression levels have been normalized with the average expression of the Gapdh endogenous control gene.*

3. c-Myc protein by western blot

Western blot was performed to detect c-Myc protein level. All cell lines showed expression of c-Myc protein by western blot (Figure 3). We found that SW620 showed the highest expression of both c-Myc compared with the expression in SW480, and SW837, suggesting that metastasis are more proliferative and aggressive than primary tumors.



▲ **Figure 3**. *c-Myc protein expression detected by western blot.*

Discussion

Currently, no effective method exists that predicts response to CRT in LARC. According to previous result (1), c-Myc could be a new biomarker to predict response to treatment in LARC patients (6).

In the present study, we analyze the c-Myc status in 3 cell lines in order to establish the best cell line model to, subsequently, carry out genome silencing of c-Myc by means of iRNA.

We identified c-Myc mutations in SW480, SW620, and SW837. The variant described in SW480 and SW620, did not report in mutation database (7). In SW837, we detected c.274G>A in c-Myc leading to an exchange of valine for isoleucine at amino acid position 92 of the protein. This missense mutation was also reported in mutation database and classified as single polymorphism. We thought that this variant could not have major effects on protein given that valine and isoleucine are branched chain amino acids, and they have a similar composition of carbons, oxygens, hydrogens, and nitrogen.

The results reported here show c-Myc is expressed in SW480, SW620, and SW837 cell lines. We found that SW620, derived from metastatic site (lymph node) of the same tumor from SW480, showed higher expression of c-Myc mRNA compared with the expression of SW480 and SW837. The c-Myc protein showed the same pattern as those observed at mRNA level. The c-Myc protein was detected in all cases, and SW620 showed greater amounts of c-Myc protein. This higher expression of c-Myc in cell lines derived from metastatic could be because the cell undergoes many genetic and epigenetic changes as it transitions to malignancy and metastatic phenotype (8).

Conclusion

In this study we characterized c-Myc (DNA, RNA, and protein) in 3 different tumoral cell lines in order to carry out genome silencing of c-Myc. Our data show that c-Myc sequence is altered in these cell lines. The metastatic SW620 presents the highest levels of c-Myc (RNA and protein), but this cell line is not the best model given that it is derived from the lymph node of the same tumor as the SW480. However, since the three cell lines showed c-Myc protein expression, we are going to silence c-Myc to establish the role of c-Myc in response to treatment in LARC patients.

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Competing interests

The authors have declared that no competing interests exist.

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