High Cytoplasmic FOXO3 Expression Associated with Poor Prognosis in Rectal Cancer Patients Received Preoperative Radiotherapy: A Study from Swedish Clinical Trial of Preoperative Radiotherapy to Big Database Analysis

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Research

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Abstract

Background: Accumulating evidence has implicated a pivotal role for FOXO3, FOXM1 and SIRT6 in cancer progression and metastasis. The majority of researches have focused on the functions of these proteins in drug resistance, but their relationships with radiotherapy (RT) response remain unclear. In this study, we examined the protein expression of FOXO3, FOXM1 and SIRT6 and their clinical significance in a Swedish rectal cancer trial of preoperative RT.

Methods: Protein expression of FOXO3, FOXM1 and SIRT6 protein was examined by immunohistochemistry in tissue microarray (n =145). Genetic analysis of FOXO3, FOXM1 and SIRT6 were performed by cBioportal and MEXPRESS database. Gene-gene network analysis was conducted using GeneMANIA. Further functional enrichment analysis was performed based on LinkedOmics and Metascape online software.

Results: The results showed that the FOXO3 and FOXM1 proteins were mainly expressed in the cytoplasm in both the normal and tumour tissues, and SIRT6 in both the cytoplasm and nucleus of the normal and tumour tissues. The FOXO3 and FOXM1 protein levels increased from the normal mucosa to the primary cancer (P < 0.001), while the SIRT6 protein decreased from the normal mucosa to the primary cancer (P < 0.001). High FOXO3 expression correlated with late TNM stage (P = 0.040) and distant metastasis (P = 0.032), as well as independently with disease free survival (RFS) in RT patients (HR = 7.948; P = 0.049; 95% CI = 1.002-63.032) but not in non-RT patients (P > 0.05). Furthermore, genetic analysis indicated that DNA methylation status contributes to the FOXO3 overexpression. Functional enrichment analysis demonstrated that FOXO3 is closely related to metabolism-related signalling pathway which is in turn associated with cancer radioresistance. Moreover, there were strong gene–gene interactions between FOXO3 and genes in metabolism-related signalling.

Conclusions: Our findings suggest that FOXO3 may be a key predictive biomarker to optimise RT for rectal cancer patients to improve prognosis.

Trial registration: Radiotherapy; 86151; Registered 17 November 1986; NCT00904813; https://clinicaltrials.gov/ct2/show/NCT00904813?term=Preoperative+Radiotherapy&cndry=SE&draw=3&rank=3.

Background

Colorectal cancer is one of the most common cancers worldwide (1). Although the treatments for colorectal cancer have been greatly improved in the last decades, one third of patients still display distant recurrence in 5 years according to the 10-year follow up of EOTRC trail (2). Preoperative radiotherapy (RT) has been commonly used in the clinics as a standard routine treatment for rectal cancer patients and has proven to have beneficial effects on lowering recurrence and increasing survival rates in the majority of clinics (3, 4). However, it has been also reported that RT can also enhance cancer migration and invasion, which results in poorer prognosis in patients (5). This indicates that the exact mechanisms underlying the
therapeutic effects of RT as the standard therapy for rectal cancer patients have not been completely elucidated. In consequence, it is pertinent to provide better evidence to further support the positive effects of the RT for rectal cancer therapy, such as biomarkers to distinguish patients who would benefit from preoperative RT and improve survival.

Forkhead box (FOX) family proteins, function mainly as transcription factors, have been extensively investigated in a number of cellular processes (6). Numerous studies have shown that two members of Forkhead family, FOXO3 and FOXM1, play extremely crucial roles in cancer progression (6). FOXO3 (also previously known as FKHL1), belonging to the FOXO subfamily, has a controversial role in the progression of cancer, and is involved in regulating the cell cycle, inhibiting proliferation and programming cell death. Our previous study has shown that the de-activation of FOXO3 may depend on the phosphorylation by protein kinase B (also called Akt) (7), and the phosphorylated forms of FOXO3 are translocated from the nucleus to the cytoplasm. FOXM1 is proven to promote cell cycle progression, DNA damage repair and cell survival (8, 9) upon DNA damage. In addition, FOXM1 can also work with FOXO3 in an axis (8); both can bind to the same downstream target genes and antagonise the functions of each other. Meanwhile, as one of the key targets of FOXO3 and FOXM1, SIRT6, as a member from nicotinamide adenine dinucleotide (NAD)+-dependent sirtuin protein family, has also attracted lots of attention. Like FOXO3 and FOXM1, SIRT6 is involved in many crucial cellular processes including metabolism, genome stability maintenance and ageing, may also promote tumorigenesis and cancer progression (10).

Previous studies have focused on the involvement of FOXM1/FOXO3/SIRT6 network in cancer development, chemoresistance and radioresistance among several cancer types, including breast, liver and bladder cancer (7, 10, 11). Hitherto, little is known about the expression patterns of FOXO3, FOXM1 and SIRT6 in rectal cancer patients with and without preoperative RT, therefore it is crucial to understand if the expression of these molecules is also involved in RT response in rectal cancer. Through investigating the patient materials from a Swedish rectal cancer clinical trial, in this study we are the first to identify FOXO3 as an independent prognostic factor in rectal cancer patients receiving RT. Our findings suggest that FOXO3 may be a reliable biomarker for optimising RT for rectal cancer patients in order to improve prognosis.

Results

Expression of FOXO3, FOXM1 and SIRT6 proteins in normal mucosa, primary cancer and lymph node metastasis from rectal cancer patients

As shown in Fig. 1A, FOXO3 was predominantly expressed in the cytoplasm of epithelial cells in the normal mucosa (left panel), primary cancer (middle panel) and lymph node metastasis (right panel). In the non-RT group, high levels of FOXO3 expression were detected in primary cancer (90%) when compared with distant normal mucosa (14%) and adjacent normal mucosa (18%) respectively (both P < 0.001), and there were no statistical significant changes in FOXO3 expression levels from primary cancer
(90%) to metastasis (82%) (Figure. 1B). In the RT group, the proportion of cells with high FOXO3 expression was significantly increased in primary cancer (77%) compared with distant normal mucosa (9%) and adjacent normal mucosa (6%), respectively (both P < 0.001), while it was reduced from primary cancer (77%) to metastasis (64%) (P = 0.044; Fig. 1B).

FOXM1 was found mainly in the cytoplasm of the epithelial cells in the normal mucosa, primary cancer and lymph node metastasis (Fig. 1C). In the non-RT group, the proportion of cells with high FOXM1 expression was significantly augmented in primary cancer (87%) compared with distant normal mucosa (48%) and adjacent normal mucosa (47%) respectively (both P < 0.001), but there were no statistically significant changes going from primary cancer (87%) to metastasis (77%) (Fig. 1D). However, there were no significant changes in FOXM1 expression among distant normal mucosa (68%), adjacent normal mucosa (75%), primary cancer (75%) and metastasis (58%) in the RT patients (all P > 0.05).

SIRT6 was found to express in both the cytoplasm and nucleus of the epithelial cells in the normal mucosa, as well as cancer cells in primary cancer and lymph node metastasis (Fig. 1E). Since SIRT6 is considered to function predominantly in the nucleus, the nucleic staining was evaluated further. In the non-RT group, the frequency of nuclear SIRT6 expression was decreased significantly from the distant normal tissue (39%) to adjacent normal tissue (30%) and to primary cancer (9%) (both P < 0.001), and there were no significant changes in nuclear SIRT6 expression between primary cancer (9%) and metastasis (11%). In the RT group, the frequency of high SIRT6 expression was decreased significantly from distant normal mucosa (56%), to adjacent normal mucosa (31%) and primary cancer (12%) (both P < 0.001), and to metastasis (5%) (all P < 0.05) (Fig. 1F).

**Relationship of FOXO3, FOXM1 and SIRT6 protein expression with clinicopathological variables in rectal cancer patients**

We next investigated the significance of FOXO3 and FOXM1 expression in the primary cancer in relation to various clinicopathological factors (Table 1). FOXO3 expression was found to be significantly associated with the late TNM stages (P = 0.040), recurrence (local + distant recurrence, P = 0.018) and distant recurrence (P = 0.032) in RT patients, and also recurrence (P = 0.028) in non-RT patients. There was no relationship between FOXO3 and gender, age, differentiation or local recurrence (all P > 0.05, Table 1) in either RT or non-RT patients. The cytoplasmic FOXM1 expression was not related to any of the above-mentioned clinicopathological variables in either RT or non-RT patients (P > 0.05). SIRT6 expression was also not correlated with any of the above-mentioned clinicopathological variables in either RT or non-RT patients (P > 0.05).
| Variables                          | FOXO3 expression | FOXO3 expression |
|-----------------------------------|------------------|------------------|
|                                   | Non-RT           | RT               |
|                                   | Low (%)          | High (%)         | p-value | Low (%)          | High (%)         | p-value |
| Gender                            | 0.265            | 0.816            |
| Male                              | 3 (7) 39 (93)    | 8 (22) 29 (78)   |         |
| Female                            | 5 (15) 28 (85)   | 4 (19) 17 (81)   |         |
| Age (years)                       | 0.145            | 0.244            |
| ≤ 69                              | 6 (16) 32 (84)   | 9 (26) 26 (74)   |         |
| > 69                              | 2 (5) 35 (95)    | 3 (13) 20 (87)   |         |
| Tumour stage                      | 0.096            | 0.040            |
| I                                 | 3 (14) 18 (86)   | 7 (39) 11 (61)   |         |
| II                                | 4 (21) 15 (79)   | 4 (19) 17 (81)   |         |
| III                               | 1 (3) 34 (97)    | 1 (5) 18 (95)    |         |
| Differentiation                   | 0.129            | 0.743            |
| Well                              | 1 (50) 1 (50)    | 0 1 (100)        |         |
| Moderately                        | 5 (8) 56 (92)    | 10 (23) 34 (77)  |         |
| Poorly                            | 2 (17) 10 (83)   | 2 (15) 11 (85)   |         |
| Recurrence                        | 0.028            | 0.018            |
| No                                | 7 (18) 31 (82)   | 11 (31) 25 (69)  |         |
| Yes                               | 1 (3) 36 (97)    | 1 (5) 21 (95)    |         |
| Local recurrence                  | 0.093            | 0.290            |
| No                                | 8 (14) 49 (86)   | 12 (22) 42 (78)  |         |
| Yes                               | 0 18 (100)       | 0 4 (100)        |         |
| Distant recurrence                | 0.124            | 0.032            |
| No                                | 7 (15) 40 (85)   | 11 (29) 27 (71)  |         |
| Yes                               | 1 (4) 27 (96)    | 1 (5) 19 (95)    |         |
We further analysed the relationship between FOXO3 expression and survival in non-RT and RT patients, respectively. In the non-RT group, there was no statistically significance between the high and low FOXO3 expression regarding cancer-specific survival (CSS) (P = 0.119; Fig. 2A). However, in the RT group, patients with high FOXO3 expression was found to have poor CSS compared with patients with low FOXO3 expression (P = 0.047, Fig. 2B). Moreover, those patients with high FOXO3 expression had poor disease-free survival (DFS) in both the non-RT group (P = 0.049, Fig. 2C) and RT group (P = 0.022, Fig. 2D). The significance between high and low FOXO3 expression in DFS still existed, after adjusting for gender, age, TNM stage, tumour differentiation, surgical type and resection margin in the RT group (Table 2). The patients with high FOXO3 expression were 7.948 times more likely to have disease recurrence than patients with low FOXO3 expression (HR, 7.948; P = 0.049; 95% CI, 1.002–63.032). In addition, we found that the female patients were less likely to relapse compared to male patients in multivariate analysis (HR, 0.190; P = 0.014; 95% CI, 0.051–0.711). There was no significance in the non-RT group regarding either univaraint or multivariant factors mentioned above; however, significances were found in multivaraint analysis in CSS (HR, 8.717; P = 0.006; 95% CI, 1.842–41.261) and DFS (HR, 4.854; P = 0.030; 95% CI, 1.162–20.275) regarding mucinous versus non-mucinous differentiation. For the study above, we accounted only for tumours stages I–III, since stage IV may affect the prognosis beyond RT and biomarkers.

**Table 2.** Univariate and multivariate analysis of FOXO3, gender, age, stage, differentiation, surgical type and resection margin in relation to survival of rectal cancer patients in RT group.

| Variables† | CSS | DFS |
|------------|-----|-----|
|             | Univariate analysis | Multivariate analysis | Univariate analysis | Multivariate analysis |
| FOXO3 expression | | | | |
| (high vs. low) | HR (95%CI) | p-value | HR (95%CI) | p-value | HR (95%CI) | p-value | HR (95%CI) | p-value |
|              | 5.434 | 0.047 | 4.236 | 0.174 | 7.344 | 0.022 | 7.948 | 0.049 |
| Gender | | | | |
| (female vs. male) | 0.507 | 0.154 | 0.396 | 0.184 | 0.556 | 0.154 | 0.190 | 0.014 |
| Age | | | | |
| (>69 vs. ≤69) | 0.801 | 0.598 | 0.640 | 0.427 | 0.714 | 0.374 | 0.594 | 0.282 |
| TNM stage | | | | |
| (III vs. I–II) | 6.130 | 0.001 | 6.500 | <0.001 | 6.945 | <0.001 | 3.343 | <0.001 |
| Differentiation | | | | |
| (mucinous vs. non-mucinous) | 1.468 | 0.000 | 1.000 | 1.091 | 0.895 | 0.000 | 1.000 |
| Surgical type | | | | |
| (anterior resection vs. rectum amputation) | 0.828 | 0.644 | 0.814 | 0.895 | 0.576 | 0.531 | 0.261 |
| Resection margin | | | | |
| (positive vs. negative) | 1.391 | 0.65 | 0.435 | 0.496 | 1.064 | 0.933 | 0.181 | 0.157 |

*Relationship of FOXO3 protein expression with biological factors in rectal cancer patients*
As shown in Table 3, the relationships of FOXO3 expression with biological factors have previously been examined on the same patient cohort at our laboratory. In the RT group, FOXO3 expression in the primary tumours was positively correlated with FOXM1 (P = 0.003) and cytoplasmic phospho-NF-κB at Serine 536 (P = 0.049). There was no statistical significance between FOXO3, and SIRT6, p53, p73, survivin, Cox-2 or PPAR-delta (all P > 0.05). In the non-RT patients, FOXO3 was also positively correlated with NF-κB expression (P = 0.04). However, there were no significant correlations between FOXO3 and the other biological factors mentioned above (P > 0.05).
Table 3
FOXO3 expression in the primary rectal tumour in relation to biological variables.

| Variables | FOXO3a expression | FOXO3a expression | p-value | FOXO3a expression | FOXO3a expression | P-value |
|-----------|-------------------|-------------------|---------|-------------------|-------------------|---------|
|           | Low (%)           | High (%)          |         | Low (%)           | High (%)          |         |
| NFκBp65   | 0.653             | 0.049             |         | 0.653             | 0.049             |         |
| Low       | 0                 | 2 (100)           |         | 0                 | 3 (50)            |         |
| High      | 7 (3)             | 69 (97)           |         | 9 (6)             | 46 (94)           |         |
| FOXM1     | 0.317             | 0.003             |         | 0.317             | 0.003             |         |
| Low       | 0                 | 10 (100)          |         | 7 (50)            | 7 (50)            |         |
| High      | 6 (14)            | 59 (86)           |         | 6 (13)            | 40 (87)           |         |
| p53       | 0.951             | 0.097             |         | 0.951             | 0.097             |         |
| Low       | 7 (88)            | 60 (88)           |         | 11 (20)           | 44 (80)           |         |
| High      | 1 (12)            | 8 (12)            |         | 3 (50)            | 3 (50)            |         |
| p73       | 0.163             | 0.068             |         | 0.163             | 0.068             |         |
| Negative  | 8 (14)            | 48 (86)           |         | 6 (35)            | 11 (65)           |         |
| Positive  | 0                 | 12 (100)          |         | 4 (29)            | 27 (71)           |         |
| Survivin  | 0.847             | 0.505             |         | 0.847             | 0.505             |         |
| Low       | 5 (12)            | 36 (88)           |         | 6 (22)            | 21 (78)           |         |
| High      | 1 (10)            | 9 (90)            |         | 3 (33)            | 6 (67)            |         |
| Cox2      | 0.463             | 0.175             |         | 0.463             | 0.175             |         |
| Low       | 2 (8)             | 22 (92)           |         | 3 (14)            | 19 (86)           |         |
| High      | 5 (15)            | 29 (85)           |         | 7 (30)            | 16 (70)           |         |
| PPAR-delta| 0.767             | 0.069             |         | 0.767             | 0.069             |         |
| Low       | 6 (10)            | 56 (90)           |         | 14 (28)           | 36 (72)           |         |
| High      | 1 (7)             | 13 (93)           |         | 0                 | 9 (100)           |         |
| Sirt6     | 0.384             | 0.861             |         | 0.384             | 0.861             |         |
| Negative  | 7 (10)            | 64 (90)           |         | 12 (22)           | 42 (78)           |         |
| Positive  | 0                 | 7 (100)           |         | 2 (25)            | 6 (75)            |         |
Table 4

Characteristics of patients and tumours of FOXO3.

| Characteristics     | Non-RT (%) | RT (%) | P-value |
|---------------------|------------|--------|---------|
| **Gender**          |            |        | 0.502   |
| Male                | 45 (57)    | 40 (63)|         |
| Female              | 34 (43)    | 24 (37)|         |
| **Age (years)**     |            |        | 0.371   |
| ≤69                 | 41 (52)    | 38 (59)|         |
| >69                 | 38 (48)    | 26 (41)|         |
| **Stage**           |            |        | 0.268   |
| I                   | 21 (27)    | 18 (28)|         |
| II                  | 19 (24)    | 21 (33)|         |
| III                 | 35 (44)    | 19 (30)|         |
| IV                  | 4 (5)      | 6 (9)  |         |
| **Differentiation** |            |        | 0.773   |
| Well                | 2 (3)      | 1 (2)  |         |
| Moderately          | 63 (80)    | 49 (76)|         |
| Poorly              | 14 (17)    | 14 (22)|         |
| **Number**          |            |        | 0.518   |
| Single              | 71 (86)    | 52 (84)|         |
| Multiple\(^a\)      | 10 (12)    | 10 (16)|         |
| Unknown             | 2 (2)      | 0 (0)  |         |
| **Surgical type**   |            |        | 0.044   |
| Rectal amputation   | 43 (54)    | 24 (37)|         |
| Anterior amputation | 36 (46)    | 40 (63)|         |
| **Resection margin**|            |        | 0.918   |
| Negative            | 75 (95)    | 61 (95)|         |
| Positive            | 4 (5)      | 3 (5)  |         |
| **To anal verge (cm)**|          |        |         |
| Mean                | 7.341      | 8.656  |         |
Gene-gene interaction networks among FOXO3, FOXM1 and SIRT6 in colorectal cancer

Furthermore, we constructed the gene-gene network and function analysis using the GeneMANIA database. As showed in Figure 3, FOXO3, FOXM1, SIRT6 and genes in NF-κB pathways have close relationships in the gene-gene interaction networks, such as co-expression, shared protein domains, co-localization, pathway and genetic interaction. Evidently, they have genetic and physical interactions, and are involved similar pathways with other genes. Moreover, they also share similar protein kinases and diacylation as well as DNA repair and cell cycle G2/M transition.

The genetic alteration of FOXO3, FOXM1 and SIRT6 in colorectal cancer

In order to understand comprehensively the expression profiles of FOXO3, FOXM1 and SIRT6 in colorectal cancer, we analysed their genetic alterations by cBioPortal. The mutation ratios of FOXO3, FOXM1 and SIRT6 were found to be 7%, 6% and 2.9%, respectively (Figure 4A). And their alteration frequencies in different subtypes of colorectal cancer were also examined (Figure 4B). The analysis revealed that higher frequencies of FOXO3, FOXM1 and SIRT6 mRNA overexpression were commonly found in in various subtypes of colorectal cancer. The FOXM1 mRNA overexpression was associated with relatively higher copy number amplification. The details concerning mutations of FOXO3 and FOXM1 in colorectal cancer were also analysed and shown in Figure 4C. However, the analysis also demonstrated that the upregulation of FOXO3 and SIRT6 expression was not resulted from gene amplification.

DNA methylation status of FOXO3 in colorectal cancer

Dysregulation of the FOXO3 can be due to its DNA methylation status. As a result, further evaluation for DNA methylation status of FOXO3 gene in colorectal cancer was carried out and shown in Table S1. There was a negative correlation between mRNA expression and DNA methylation of FOXO3 (R ≥ 0.5, P < 0.05). Specific methylation site analyses of FOXO3 were performed additionally based on MEXPRESS dataset. This indicated that distinct DNA methylation sites might regulate the FOXO3 expression.

Functional analysis of FOXO3-related genes in colorectal cancer

To uncover the mechanisms underlying the significant prognostic value of FOXO3 in colorectal cancer, we further explored the possible molecular functions of FOXO3 based on TCGA datasets. The circus plot in Figure 5A and the volcano plot in Figure 5B showed the genomic location of FOXO3 and all FOXO3-associated genes in colorectal cancer. As shown in Figure 5C and 5D, the heat maps showed the genes positively and negatively associated with FOXO3 in colorectal cancer, respectively.

GO (Gene Ontology) enrichment analysis indicated that the FOXO3-associated genes were significantly linked to several metabolism-related biological processes (Figure 6A and Supplementary table 3), such as generation of precursor metabolites and energy (gene ratio = 23/522, Log(p-value) = -16.627), ATP
metabolic process (gene ratio = 18/311, Log (p-value) = -15.068) and ribonucleotide metabolic process (gene ratio = 18/311, Log(p-value) = -15.068). To further capture the internal associations among the terms, the top 20 enriched clusters were rendered as a network plot using Metascape online tools. A Kappa similarity > 0.3 was considered as a good connection (Figure 6B and 6C). Consistently, as shown in Figure 6D and Table S2, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis also demonstrated that FOXO3 was highly associated with metabolism-related signal pathways: Carbon metabolism (gene ratio = 5/114, Log(p-value) = -3.940215054) and Metabolism of xenobiotics by cytochrome P450 (gene ratio = 3/76, Log(p-value) = -2.407652205). Moreover, a gene–gene interaction network performed by GeneMANIA database also showed that there are several interactions between FOXO3 and genes involved in cell metabolism, including co-expression, co-localization and genetic interaction (Figure 6E).

**Discussion**

Our study is the first to concentrate on the relationship between high cytoplasmic expression FOXO3 and tumour progression in rectal cancer patients with preoperative RT. FOXO3 protein expression was significantly higher in primary tumours compared to normal tissues regardless of RT or non-RT status. Furthermore, higher FOXO3 expression in primary tumour was significantly associated with late TNM stages and distant recurrence in RT patients. Even more importantly, the patients whose tumours expressed high FOXO3 with RT had a worse prognosis compared to patients with tumours having low FOXO3 expression, independently of gender, age, TNM stage, tumour differentiation, surgical type and resection margin. A similar result has previously been shown in bladder cancer patients [20]. Our findings suggest that FOXO3 can be a potential prognostic biomarker for preoperative RT-associated survival in rectal cancer patients.

Our further genetic analyses indicate that the DNA methylation status is involved in misregulation of FOXO3 expression in colorectal cancer. It is generally accepted that DNA methylation is a major epigenetic process that plays a critical role in different stages of evolution and disease development, including cancer progression. A prior study reported that FOXO3 expression was upregulated in breast cancer due to DNA hypomethylation (12). In agreement, a genome DNA methylation analysis of active pulmonary tuberculosis also found that the patients with MRPS18B/FOXO3 hypomethylation showed lower than one-year survival (13). Overall, our findings suggest that DNA hypomethylation is involved in the FOXO3 upregulation and the associated poor prognosis in colorectal cancer patients.

To explore further the FOXO3 function networks, we analysed the relationships of FOXO3 expression with other biological factors examined previously in the same patient cohort from our laboratory. Interestingly, our results indicated that FOXO3 expression in primary tumours was positively correlated with FOXM1 and the cytoplasmic phospho-NF-κB in the RT-group. Because of pleiotropic oncogenic role of NF-κB in a variety of cancer types, it is logically to speculate that further unappreciated functions of FOXO3 in cancer progression exist and the detailed mechanisms require further investigation.
The functions of FOXO3 in cancer progression, RT response and prognosis are controversial. In general, the FOXO3 has been considered as a suppressor in primary tumour growth through promoting apoptosis and/or restricting cell cycle progression. However, phosphorylation by kinases, particularly PI3-K/PKB signalling pathway, ERK, IKB kinase and serum and glucocorticoid-regulated kinase can promote FOXO3 nuclear to cytoplasmic shuttling, which may subsequently alter its normal biological functions in cancer progression. In chronic myeloid leukaemia, although FOXO3 activation can provoke Bim-induced apoptosis in Bcr-Abl-expressing cells through treatment with the Bcr-Abl inhibitor STI571 (14), continuous FOXO3 activation induced by doxorubicin have also been found to lead to drug resistance and cell survival via stimulating PIK3CA and ABCB1 expression (15). Conversely, there is also evidence in colorectal cancer that knockdown of FOXO3 reduced MEK/MAPK phosphorylation and anchorage-independent growth, significantly decreasing the sensitivity to cetuximab in cells harbouring mutant, but not wild-type, KRAS (16). As a candidate therapeutic strategy in pancreatic ductal adenocarcinoma, FOXO3 inhibition has been shown to suppress CD44 expression and cancer stem cell properties via the signalling axis of FOXO3/LKB1/AMPK/PGC-1β/PDHA1/CD44 (17, 18). Collectively, these functions of FOXO3 in cellular detoxication (19), the development of drug-resistance (15, 20) and the feedback-regulation on PI3K/PKB-activity (21) also support a tumour promoting role for FOXO3. Nevertheless, irrespective of whether FOXO3 functions as an oncogene or a tumour suppressor, it is reasonable to propose that FOXO3 is crucial molecule in various aspects of cancer development, especially in drug sensitivity and resistance. In consequence, it is important to understand its role and expression in cancer treatment in order to uncover treatment strategies for overcoming chemotherapeutic drug resistance.

As mentioned previously, FOXM1 has been proven to be involved in a number of crucial processes in oncogenesis and cancer progression (6), such as stem cell expansion, epithelial-mesenchymal transition, and drug resistance (11). Patients with high levels of FOXM1 expression have significantly poorer survival compared to patients with low FOXM1 expression, which is in concordance with previous studies in lung cancer [21] and glioblastoma [22] radioresistance pathway is a central actor of GBM treatment resistance and a key target to inhibit in the aim to increase the sensitivity of GBM to the RT. Multivariate analysis indicated that FOXM1 is an independent prognostic factor for disease-free survival in male breast cancer (6, 22). At odds with these results, we found that there was no significant correlation between FOXM1 and survival in our patient samples, which may due to the differences in patient samples/resources available or/and cancer types.

SIRT6 has also been observed to be a downstream target of FOXO3. SIRT6 has been reported to be able to restrict cancer survival and inhibit tumour prognosis via impairing in collaboration with HIF1α and p53 (23) and repair DNA damage (10). In our patient samples, nuclear SIRT6 expression decreased from normal tissues to tumours and metastases in the lymph nodes. Even in the patients with non-small cell lung cancer, low nuclear and high cytoplasmic SIRT6 expression has been found to be associated with poor survival (23). However in our study there was no significant correlation between SIRT6 and survival in rectal cancer, indicating that SIRT6 may play different roles in distinct cancer types, and its expression might not directly affect rectal cancer progression in relation to RT.
In the present study, we found that FOXO3 and FOXM1 expression in the cytoplasm of both the normal mucosa and cancer tissue. However, SIRT6 protein was expressed in the both cytoplasm and nucleus of the normal mucosa and cancer tissue. The expression of FOXO3, FOXM1 and SIRT6 protein was significantly different between normal mucosa, primary tumour and lymph node metastasis in RT and/or non-RT patients. Moreover, FOXO3 expression was statistically related to TNM stages, recurrence and prognosis, as well as NF-κB p65 in RT patients (23). Further enrichment analysis based on TCGA dataset also demonstrated that FOXO3 was involved in several metabolism-related signalling in colorectal cancer, which was reported to play a crucial role in radioresistance (24). These findings strongly suggest that FOXO3, FOXM1 and SIRT6 play various roles in rectal cancer, and FOXO3 was the core molecule involved in the rectal cancer progression, RT response and prognosis (Fig. 7).

Conclusions

In the present study, high FOXO3 expression was strongly associated with late TNM stages, distant recurrence, and poor prognosis, independently of gender, age, TNM stage, tumour differentiation, surgical type and resection margin in RT patients, but not in non-RT patients. In conclusion, FOXO3 is the key biomarker for predicting RT response and prognosis in rectal cancer patients.

Abbreviations

RT: radiotherapy; RFS: disease free survival; FOX: Forkhead box; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; cBioPortal: cBio Cancer Genomics Portal.

Materials And Methods

Rectal cancer patients and materials

Patients were from the South-East Swedish Health Care region and participated in the randomized Swedish Rectal Cancer Trial of preoperative RT between 1987 and 1990 (Swedish Rectal Cancer Trial, 1997 (3)). Every participant signed the informed consent. The patient cohort of FOXO3 included 143 primary rectal adenocarcinomas, 124 normal mucosa specimens (112 corresponding to the primary cancer, i.e., normal mucosa and primary cancer from the same patient) that were histologically free from cancer and taken from the margin of distant surgical resection, and 50 lymph node metastases (47 corresponding to the primary cancer, also in the irradiation field). Of the 143 patients (median age, 69 years), 79 underwent surgery alone and 64 received RT followed by tumour resection. RT was given with 25 Gy in 5 fractions within a median of 7 days (range, 4–12 days). Surgery was then carried out in a median of 3 days (range, 0–11 days) after RT. None of the patients received preoperative or adjuvant chemotherapy. The mean follow-up period was 105 months (range, 0-309 months), and information on local and distant recurrence; disease free survival and overall cancer-specific survival (OS) were obtained from patient medical records. The patient cohort of FOXM1 included 141 primary rectal adenocarcinomas, 103 normal mucosa specimens (85 corresponding to the primary cancer, i.e., normal
mucosa and primary cancer from the same patient) that were histologically free from cancer and taken from the margin of distant surgical resection, and 45 lymph node metastases (41 corresponding to the primary cancer, also in the irradiation field). Of the 141 patients (median age, 69 years), 77 underwent surgery alone and 64 received RT followed by tumour resection. All the rest of information is the same as FOXO3. The patient cohort of SIRT6 included 145 primary rectal adenocarcinomas, 120 normal mucosa specimens (84 corresponding to the primary cancer, i.e., normal mucosa and primary cancer from the same patient) that were histologically free from cancer and taken from the margin of distant surgical resection, and 49 lymph node metastases (45 corresponding to the primary cancer, also in the irradiation field). Of the 145 patients (median age, 69 years), 80 underwent surgery alone and 75 received RT followed by tumour resection. All the rest information is the same as FOXO3. All the characteristics of the patients and tumours of these three factors are presented in Table 4 (FOXO3), Table S3 (FOXM1) and Table S4 (SIRT6).

**Tissue samples and immunohistochemistry (IHC)**

Expression of FOXO3, FOXM1 and SIRT6 protein was examined by IHC in 4 µm tissue microarray sections from paraffin-embedded surgical specimens. Sample sections were deparaffinized by immersing the slides twice in 100% xylene at room temperature for 10 minutes each. This was followed by incubating twice in 100% ethanol for 10 minutes each, and rehydrating with decreasing concentrations of ethanol (90% and 70%; vol/vol in water, 10 minutes each) before a final 5-minute incubation in water. Antigen retrieval was carried out in a target retrieval citrate buffer (pH 6.0) (Dako, Glostrup, Denmark) at 95 °C for 15 minutes. The sections were allowed to cool for 15 minutes and rinsed with phosphate-buffered saline (PBS) followed by incubation in 3% H\textsubscript{2}O\textsubscript{2}-methanol for 5 minutes to block the activity of endogenous peroxidase. After being washed in PBS, the sections were incubated with protein block (Dako) for 10 minutes to reduce nonspecific background staining. The sections were incubated with primary antibody overnight. After being washed in PBS, the sections were incubated with a secondary antibody, Envision System Labelled Polymer-HRP Anti-Rabbit (Dako) for 25 minutes. The sections were rinsed in PBS before reacting with Liquid DAB+ (Dako) to produce coloration. Finally, the sections were lightly counterstained with haematoxylin.

The immunostaining was scored by two independent observers based on the intensity and localization. Staining intensity in normal epithelial cells or tumour cells was graded according to the following criteria: 0 (no staining); 1 (weak staining = light yellow); 2 (moderate = yellow brown); 3 (strong = brown); and 4 (very strong = dark brown). The staining patterns were graded as cytoplasmic or nuclear. In case of discrepancy, a consensus score was reached after re-evaluation. For statistical analyses, cases scored less than 2 were considered as low-expressing group, and cases scored higher than 3 as high-expressing group. The data regarding IHC expression of NF-κB, p53, p73, survivin, Cox2 and PPAR-delta was obtained at our laboratory on the same patient samples as in the present study. The used cut-off points were the same as previous corresponding publications (25–28).

**cBioPortal database analysis**
The cBio Cancer Genomics Portal (cBioPortal) (http://www.cbioportal.org/) provides visualization tools for more than 5,000 tumour samples from 232 cancer studies in the TCGA database (29). In this study, the Colorectal Adenocarcinoma (TCGA, Firehose Legacy, n = 640) cohort was analysed to explore the genetic alterations of FOXO3, FOXM1 and SIRT6.

**MEXPRESS tool analysis**

The MEXPRESS tool (https://mexpress.be) is a user-friendly online tool visualizing TCGA data, contains the information on mRNA expression, DNA methylation, clinical data as well as the relationships among these parameters (30). The detail methylation locations of FOXO3 in colorectal cancer were assessed using the MEXPRESS.

**GeneMANIA analysis**

GeneMANIA (http://www.genemania.org) is a friendly web server for deriving hypotheses based on gene functions (31). GeneMANIA was adopted to conduct a gene-gene interaction network for FOXO3, FOXM1 and SIRT6.

**The Cancer Regulome tools and data analysis**

The Cancer Regulome tools and data (http://explorer.cancerregulome.org/) from the TCGA dataset were performed to draw circus plots to show the genomic location of FOXO3 and its related-genes in colorectal cancer (n = 621). Spearman correlation was used to show the pairwise correlation between two genes. The circus plots only display the genes with $P$-values > log10.

**Functional enrichment analysis**

The Spearman correlation analysis was conducted by the LinkedOmics database (http://www.linkedomics.org/) (32). Spearman's correlation coefficient exceeding 0.4 indicates a good correlation between FOXO3 and its related genes. Metascape online software (http://metascape.org) was used to construct the interaction network of enrichment terms. All analyses were performed with default software parameters (33).

**Statistical analyses**

All statistical analyses were performed using STATISTICA software package (version 12.0; STATSOFT Inc., Tulsa, OK). McNemar’s or Person $\chi^2$ test was used to examine the significance of the differences in FOXO3/FOXM1/SIRT6 expression among normal mucosa, primary tumour and lymph node metastasis, as well as the association of FOXO3/FOXM1/SIRT6 expression with clinicopathological or biological variables. The survival curves were plotted using Kaplan–Meier analysis and the differences between the curves were calculated by Log rank test. Univariate and multivariate analyses were performed by Cox proportional hazards regression analysis (likelihood ratio test). All tests were two sided and $P$-values < 0.05 were considered statistically significant.
Declarations

Ethics approval and consent to participate:

This study was approved by the institutional ethics committee at Linkoping University. The informed consent was signed by each participant.

Consent for publication:

The consent forms were signed by every participant and will be provided upon request.

Availability of data and material:

The data and materials used or analysed during the current study are available.

Competing interests:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Authors' contributions:

WC and NX collected data, preformed analysis, interpreted the results, and wrote the manuscript. EWFL, participated the project design and provided antibodies, VHS, performed immunohistochemistry, NL, HZ, XFS, designed the project, and supervised/revised the manuscript. All authors read and approved the final manuscript.

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