RanGTPase is transcriptionally regulated by Myc and is required for Myc-mediated cancer progression

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Key Words: RanGTPase, metastatic progression, Myc, Survival.

Short title for running head: Myc promotes cancer via Ran

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Abstract

Background

Ran is required for cancer cell survival *in vitro* and human cancer progression. However, the molecular mechanisms for Ran overexpression in cancers are largely unknown.

Methods

The effect of Myc on Ran expression *in vitro* was investigated by Western blot, Chromatin-immunoprecipitation and luciferase reporter assays. The biological effect of Myc and Ran expression in cancer cells were investigated by soft-agar, cell adhesion and invasion assays. The correlations between Myc and Ran as well as patient survival were investigated in 14 independent patient cohorts (n = 2430).

Results:

Myc binds to the upstream sequence of the *Ran* gene and transactivates Ran promoter activity. Overexpression of Myc up-regulates the expression level of Ran, while knockdown of Myc down-regulates the expression level of Ran. Myc or Ran overexpression in breast cancer cells results in enhanced biological properties associated with cancer progression and metastasis, phenocopying each other. Knockdown of Ran reverses the effect induced by Myc overexpression in breast cancer cells. Our clinical data strongly supports our *in vitro* findings. Positive correlation between Myc and Ran expression was revealed in 288 breast cancer and 102 lung cancer specimens. This result is supported by the microarray analysis in 1454 breast cancer patients and 586 lung

cancer patients from 12 different cancer patient datasets. Moreover, Ran expression levels differentiate better or poorer survival in Myc overexpressing patients.

Conclusion

Our results suggest that Ran is required for and is a potential therapeutic target of Myc-driven cancer progression in both breast and lung cancers.

Introduction

Ran GTPase (Ran) is a small GTPase belonging to the Ras superfamily. Unlike other members, Ran lacks a cysteine residue at its C-terminus and hence does not undergo prenylation. Instead, it has an acidic C-terminal sequence –DEDDDL- which is essential for its cellular functions, including nucleocytoplasmic transportation, mitosis and centrosome reduplication [1-3]. The importance of Ran in tumorigenesis and as a cancer therapeutic target has been described in recent studies. Ran is overexpressed in cancer cell lines and tumor tissues at both mRNA and protein levels compared to their normal counterparts [4]. Ran expression is required for mitosis of cancer cells but not for normal cells [5, 6]. Cancer cells with K-Ras activating mutations are more dependent on Ran expression than their K-Ras wild-type counterparts [7, 8]. Ran expression is required for the survival of cancer cells with hyperactivation of the PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways [8]. Ran is also a poor prognostic marker in breast, lung, ovarian cancers and renal cell carcinoma [9-11]. In addition, we and others have shown that Ran over-expression may play a role in the metastatic development of breast and lung cancers, highlighting a novel role of Ran in cancer progression [12, 13].

Myc is an important oncogene that drives tumorigenesis by modulating various biological responses through transcriptional activation of its target genes [14]. Recently, Myc expression has been shown to associate with a "poor prognosis" signature, which is also associated with the development of distant metastases [15, 16]. Amongst other genes, Ran has been shown to be up-regulated by Myc in a rat cDNA microarray study [17]. Indeed, *in silico* prediction of transcription factor binding sites using Ran promoter sequence also revealed two potential Myc binding E-box elements (Supplementary

Figure 1). Given the important roles of Ran in cancer development and progression, it is important to study the transcriptional regulation of Ran expression, which may in turn explain its increased expression in cancer patients with poorer prognosis [8].

Methods and Materials

Cell culture conditions

Immortalized human breast epithelial cell line MCF10A and its Ras-transformed derivative MCF10AT (from Karmanos Cancer Center, Detroit, MI, USA) were maintained in DMEM/F-12 containing 5% horse serum, 10μg/ml insulin, 20ng/ml EGF, 100ng/ml choleratoxin and 0.5μg/ml hydrocortisone. Viral Packaging cell lines 293T (ATCC) and Phoenix Ampho (a gift from Nolan Laboratory, Stanford University, CA, USA) were maintained in DMEM containing 10% fetal bovine serum. Breast cancer cell lines MCF-7 (ATCC) and MDA MB 231 (ATCC), and lung cancer cell line A549 (ATCC) were cultured in DMEM containing 10% fetal bovine serum. Breast cancer cell line T47D (ATCC) was maintained in RPMI1640 containing 10% fetal bovine serum.

5' Rapid Amplification of cDNA Ends (RACE) assay

5' RACE assay was performed according to the manufacturer's instructions (Roche, Burgess Hill, UK) using the following 3 Ran promoter specific primers (RPSP):

RPSP-1: 5'-CAGGTCATCATCCTCATCCGG-3'

RPSP-2: 5'-TGTTGCCACACAACACAATG-3'

RPSP-3: 5'-CAAGGTGGCTACATACTT-3'

Plasmid

Ran cDNA was amplified by Phusion® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK) using total cDNA from MDA MB 231 cells, the forward primers RancdsF (5'-ATT GCG CTT CCG CCA TCT TT-3') and RancdsR (5'-GCT

CCA GCT TCA TTC TCA CA-3'), and cloned into pBabe-puro retroviral over-expression vector. pBabe-Myc-zeo was a gift from Prof. R. Weinberg and pRetrosuper shMyc from Prof. M. Eilers. pLKO.1-shRan1 (CCGGGCACAGTATGAGCACGACTTACTCGAGTAAGTCGTGCTCATACTGTGC TTTTTTG) and pLKO.1-shRan4 (CCGGCAGTTCAAACTTGTATTGGTTCTCGAGAACCAATACAAGTTTGAACTG TTTTTTG) were obtained from Sigma-Aldrich (Dorset, UK). Ran promoter (718 base pairs upstream of the transcription start site) was amplified using Phusion® Hot Start High-Fidelity DNA Polymerase and cloned into pGL3 basic reporter construct (Promega, Southampton, UK).

Generation of cell lines

Transfection was performed using GeneJuice® (Promega) according to the manufacturer's instruction. MCF10A pBabe-vector and MCF10A pBabe-Myc cells were generated by stable transfection by selecting cells in the appropriate amount of antibiotics. MCF10a-vector and MCF10A-Ran cells were generated by retroviral infection of pBabe-vector and pBabe-Ran plasmids, respectively. MDA MB 231-shScr (Scramble) and MDA MB 231-shcMyc cells were generated by retroviral infection of pRetrosuper-shScr and pRetrosuper-shcMyc, respectively. MDA MB 231-shScr and MDA MB 231-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan4, respectively, as previously described [8].

Western Blot

Western Blotting was performed as previously described [8]. Antibodies for Ran (Millipore, Watford, UK) were used at 1:1000 dilution, for Myc (Santa Cruz Biotechnology, CA) were used at a dilution of 1:1000 and for Actin (Sigma-aldrich) were used at a dilution of 1:5000.

Measurement of Ran activity

Ran activity was measured using Ran activation assay kit according to the manufacturer's instructions (Cell Biolabs, San Diego, CA). Briefly, GTP bound Ran was pulled down by using RanBP1 PBD Agarose bead slurry and the pulled-down complex was then dissociated by boiling in 2x reducing SDS-PAGE sample buffer. The pull-down supernatant was then analyzed by Western blotting.

Soft agar, cell adhesion and invasion assays

These biological assays were performed as previously described [18]. For soft agar assay, 5000 suspension cells in normal medium containing 0.35% (w/v) low-melting-point agarose were overlaid onto a solidified normal medium containing 0.7% (w/v) low-melting-point agarose. Cells were incubated at 37^{o}C with 5% (v/v) CO_2 for 2-3 weeks. Colonies were visualized by staining with crystal violet and counted. For cell adhesion assay, 40000 cells in suspension were seeded per well in 96-well plate and were allowed to settle for 30 and 60 minutes. Suspended cells were removed by washing 4 times with PBS and adhered cells were then fixed with 70% (v/v) ethanol, and stained with crystal violet. The excess crystal violet was washed away by deionized water and the retained dye was extracted by 70% ethanol. The absorbance at 595nm was measured in a

microplate reader. For invasion assay, 50000 cells in serum- and phenol red-free medium were seeded into the upper layer of the transwell insert containing Matrigel (BD Biosciences, San Jose, CA). Invasion through Matrigel was allowed to take place for 24 hours with serum as the chemoattractant in the lower layer. Cells above the insert were removed and those below were fixed and stained with crystal violet. Exces dye was washed away with water and the retained dye was extracted. The absorbance at 595nm was measured. All the biological assays were performed at 48 hours post-infection, where Ran knockdown has already been demonstrated while Ran knockdown-induced cell death has not been observed (Supplementary Figure 3).

Luciferase reporter assay

Luciferase reporter assay was performed using the dual luciferase assay kit according to the manufacturer's instructions (Promega), as previously described [19].

Patients and specimens

Patients and specimens were described previously [8]. A total of 320 formalin-fixed, paraffin-embedded breast carcinoma specimens were taken from an archive which was collected between 1976 and 1986 at the Breast Unit, Royal Liverpool Hospital. The mean age was 57 years (range from 29 – 92 years). No sign of metastasis was seen in these patients prior to specimen procurement. Treatment was either mastectomy or radical mastectomy with no prior endocrine or other systemic therpy. Follow-up data was collected between 14 and 20 years after treatment had been administered. All patient data has been made confidential by using an anonymized system by the Merseyside Cancer

Registry. A total of 113 formalin-fixed, paraffin-embedded early stage non-small cell lung cancer specimens, obtained from Dublin St. James' Hospital, resected by either lobectomy or pneumonectomy were incorporated into tissue microarray for IHC staining. Among these patients, 109 had not received any form of adjuvant chemotherapy before the procurement of the specimens. Annonmized follow-up information, including patient survival, histology and TNM-staging were provided by the Northern Ireland Cancer Registry.

Immunohistochemistry

Immunohistochemical staining was performed as previously described [8] according to the manufacturer's instructions [DAKO Envision+ kit (DAKO, Ely, UK)]. Ran antibody was used at a dilution of 1:100, while Myc antibody was used at a dilution of 1:250.

Evaluation of immunohistochemical staining results

Evaluation was performed as previously described [8]. For breast cancer specimens (from Liverpool), the whole section was used for staining, and the percentage of stained carcinoma (both nuclei and cytoplasm) cells was recorded from at least 10 fields of duplicate histological sections at 200x magnification by two independent observers and the average result obtained. For analysis, the tumors were divided into two catagorial groups of which < 1% carcinoma cells stained was classified as negative immunohistochemical staining, while > 1% carcinoma cells stained was classified as positive immunohistochemical staining. For lung cancer specimens (from Dublin), which have been incorporated into tissue microarray (TMA), the nuclear and cytoplasmic Ran,

and nuclear Myc expressions were scored from 0 to 5 arbitrarily based on the extent and intensity of the staining. All 3 cores of each specimen in the TMA were scored and the mean score for each specimen was obtained. The mean score was further classified into High and Low level expression with a cut-off point at 2.5. For overall Ran expression, tumors with high levels of both nuclear and cytoplasmic Ran staining were considered as high, tumors with high level of nuclear and low level of cytoplasmic Ran, or vice versa, were considered as intermediate and tumors with low levels of both nuclear and cytoplasmic Ran were considered as low.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. Differences in expression levels between groups/samples were analyzed by chi-square, Fisher's exact test or Mann-Whitney U tests, where applicable. The association between the expression level and patient survival was recorded by Kaplan-Meier plots and compared by Wilcoxon-Gehan test. A p-value of <0.05 was considered significant in all statistical analyses.

Analysis of breast and lung cancer microarray data

A total of seven breast cancer data sets (GSE1456 [20], GSE2034 [21], GSE3143 [22], GSE4922 [23], GSE7390 [24], GSE11121 [25], GSE12276 [26]), each consisting of more than 150 patients were included in the analysis. A total of five lung cancer data sets (GSE3141 [22], GSE4573 [27], GSE8894 [28], GSE13213 [29] and GSE14814 [30]), each consisting of more than 90 patients were included in the analysis. The data sets were pre-processed as previously described using R and Bioconductor for normalization [8]. The combined breast cancer data set

consisted of 1454 patients, while the combined lung cancer dataset consisted of 586 patients. The patients were stratified equally into four groups based on the expression levels of Ran and Myc (each group contains 25% of the patients) for further statistical analysis. The methodology for the bioinformatics steps is provided as Sweave documentation in Supplementary information.

Results

The effect of Myc on Ran transcription

Ran has been shown to be up-regulated by Myc in a rat cDNA microarray study on identification of Myc downstream targets [17], while our *in silico* analysis of the Ran upstream sequence revealed two Myc binding E-box elements (Supplementary Figure 1). These results led us to investigate whether Myc is a transcriptional activator of Ran. As shown in Figure 1A, overexpression of Myc by 2.1-fold resulted in up-regulation of endogenous Ran protein level in the breast MCF10AT cell line by 2.3-fold. The increase in the active form of Ran (RanGTP) was also detected upon Myc overexpression in MCF10AT cells (Figure 1A, right panel; 2-fold increase). The transcriptional start site of Ran was identified by using the 5'RACE assay, yielding a specific DNA band of about 200 base-pairs as the 5'RACE PCR product (Figure 1B). By sequencing this PCR product, a cytosine residue at 68 base-pairs upstream of the translational initiation site was identified as the transcriptional start site of the *Ran* gene in MCF10AT cells (Figure 1C and Supplementary Figure 2). Based on the *in silico* analysis of the upstream sequence from the *Ran* gene and the 5'RACE assay, we cloned the putative promoter of

Ran, Ranpro, into the luciferase reporter construct, pGL3, to produce pGL3-Ranpro, by PCR amplification using MCF10AT genomic DNA. Using the dual luciferase assay, Ran promoter activity was increased by 3.5-fold when Myc was overexpressed in MCF10AT cells (Figure 1D). In MCF10AT, T47D and MCF-7 breast cancer cell lines, the Ran promoter region containing the distal E-box was enriched in Myc ChIP products (Figure 1E). ChIP assay using Myc antibody resulted in 27-33% pull-down efficiency of the Ran promoter region containing the distal E-box element in all three breast cancer cell lines tested (Figure 1E). In contrast, the Ran promoter region containing the proximal E-box element was not enriched in the same Myc ChIP products (Figure 1E). Our results suggest that Myc binds to this distal E-box element, but not to the proximal E-box element *in vivo* in these three cell lines. The activity of the Ran promoter was significantly increased when Myc was overexpressed, and this increase was abolished when the distal E-box element was mutated (Figure 1F-G). Together, our results suggest that Myc binds to the distal E-box element in the Ran promoter region to activate expression of Ran, which results in an increase in activity of Ran (RanGTP).

The cell biological properties associated with modulation of Myc and Ran expression

When Myc was overexpressed in an immortalized but otherwise normal breast epithelial cell line MCF10A, endogenous Ran expression was increased (Figure 2A). Overexpression of Myc resulted in a significant increase in growth of MCF10A cells in soft agar ($p < 0.01$; Figure 2B). In addition, knockdown of Myc in an invasive breast cancer cell line MDA MB 231 cells resulted in down-regulation of Ran (Figure 2C) and a concomitant decrease in the number of colonies formed in soft agar (Figure 2D), in cell

invasion (Figure 2E) and in cell adhesion to fibronectin (Figure 2F), which are cellular properties associated with the metastatic state *in vitro*.

Overexpression of Ran also resulted in an increased number of colonies formed by MCF10A cells in soft agar (Figure 3A and B). We have shown previously that silencing of Ran results in appreciable apoptosis, which greatly interferes with the interpretation of data for investigation of cellular properties associated with metastasis *in vitro* [8]. To avoid induction of severe apoptosis while maintaining a significant level of Ran knockdown, the viral titer was reduced and a less potent shRNA targeting Ran, shRan4 was used. This protocol resulted in a significant down-regulation of Ran in MDA MB 231 breast cancer cells without appreciably inducing apoptosis, even after 96 hours of viral infection (Supplementary Figure 3). Knockdown of Ran in invasive MDA MB 231 cells led to a decrease in colony formation of cancer cells in soft agar (Figure 3C and D), in cell adhesion to fibronectin (Figure 3E) and in cell invasion through Matrigel (Figure 3F). These results suggest that Myc and Ran can separately stimulate similar cellular properties that are associated with cancer progression and metastasis. Overexpression of Ran in MCF10AT or knockdown of Ran in MDA MB231 cells did not change the expression of Myc (Supplementary Figure 4), suggesting that the regulatory link between Myc and Ran is uni-directional.

The effect of Ran in Myc-mediated cancer cell tumorigenesis

Overexpression of Myc resulted in up-regulation of endogenous Ran in MCF10A immortalized breast epithelial cells and this effect was reversed when the cells were infected with the above shRNA targeting Ran, shRan4 (Figure 4A). Moreover, when Myc

was overexpressed in MCF10A cells, their ability to form colonies in soft-agar was completely reversed by knocking down expression of Ran (Figure 4B). We then further investigated the importance of Ran in two more breast cancer cell lines, MCF-7 and T47D, in which their tumorigenicity has been shown to be positively associated with Myc expression levels [31-34]. Knockdown of Ran in these two breast cancer cell lines (Figure 4C and E) resulted in a decrease in their ability to form colonies in the soft agar assay (Figure 4D and F). Together, these results suggest that Ran expression is important for these breast cancer cell lines to maintain their tumorigenicity *in vitro*.

The correlation between Myc and Ran expression in human breast cancers

Immunohistochemical staining for nuclear Myc protein was highly significantly associated with staining for nuclear Ran (Fisher's exact test, $p < 0.001$), with staining for cytoplasmic Ran ($p < 0.001$) and with overall staining for Ran (Figure 5A-H; $p < 0.001$).

Moreover, when the mRNA levels of Ran and Myc from seven independent breast cancer datasets were analyzed, significant positive correlations between the mRNA levels of these two genes were observed in all the seven individual datasets (Spearman's Rank test, $p < 0.05$; Figure 5I-O). When the mRNA levels were stratified into four groups evenly and the seven datasets were combined ($n = 1454$), the positive correlation between Ran and Myc mRNA levels was highly significant (Chi-square test, $p < 0.001$; Figure 5P). These results strongly demonstrate the positive association between nuclear Myc and Ran expression in patient specimens.

The correlation of Ran expression and patient survival in Myc-overexpressing breast cancers

A significant association between positive nuclear staining for Myc and a shorter survival time of the patients was observed (Wilcoxon-Gehan test, $p < 0.001$; Figure 6A). The median survival times for patients with positive and negative staining tumors for nuclear Myc were 70.13 and > 228 months, respectively. In contrast, cytoplasmic staining for Myc did not significantly associate with patient survival time (Wilcoxon-Gehan test, $p = 0.342$; Figure 6B). Using Cox univariate regression analysis, positive staining for nuclear Myc was significantly associated with an increased risk of patient death of 10.6-fold (95% CI = 5.2 – 21.8, $p < 0.001$). These results suggest that an increased expression of Myc may promote metastatic progression of breast cancer, possibly through its nuclear rather than its cytoplasmic activity.

In our cohort of breast cancer patients with positive staining for nuclear Myc, the association between positive staining for nuclear Ran and survival was highly significant ($\chi^2 = 24.085$, RR = 9.10, 95% CI = 3.35 – 24.72, $p < 0.001$; Figure 6C). In this group of patients, the median survival time for those with positive staining for nuclear Ran was 56 months while those with negative staining was 216 months. In contrast, much less difference in median survival time between positive and negative nuclear Ran staining was observed in those patients with negative staining for nuclear Myc. The median survival time for those with positive staining for nuclear Ran was 204 months and those with negative staining was 228 months (Figure 6D). Likewise, in patients with positive staining for nuclear Myc, the association between positive staining for cytoplasmic Ran and survival was significant ($\chi^2 = 9.664$, RR = 2.08, 95% CI = 1.41 – 3.08, $p = 0.002$;

Figure 6D). In this group of patients, the median survival time for those with positive staining for cytoplasmic Ran was 56 months while those with negative staining was 190 months. In contrast, much less difference in median survival time between positive and negative cytoplasmic staining for Ran was observed in those patients with negative staining for nuclear Myc. The median survival time for those with positive staining for cytoplasmic Ran was 204 months and those with negative staining was 228 months (Figure 6D). In the combined breast cancer datasets, a high level of Ran mRNA was also associated with a shorter survival time (7 independent datasets, n = 1454, $\chi^2 = 14.26$, RR = 1.35, 95% CI = 1.16 – 1.58, $p < 0.001$; Supplementary Figure 5A). Moreover, , a high level of Ran mRNA was significantly associated with a higher relative risk (n = 728, $\chi^2 = 8.30$, RR = 1.41, 95% CI = 1.12 – 1.78, $p = 0.004$; Figure 6G) in patients expressing a high level of Myc mRNA compared to those patients expressing a low level of Myc mRNA (n = 726, $\chi^2 = 4.39$, RR = 1.28, 95% CI = 1.02 – 1.61, $p = 0.036$; Figure 6H).

The correlation between Myc and Ran expression in human lung cancers In A549 lung cancer cells, overexpression of Myc also transactivates the Ran promoter activity *in vitro* (Supplementary Figure 6A). Myc expression in A549 cells was shown to be associated with an increase proliferation and tumorigenesis [35]. We also found that knockdown of Ran in this cell line resulted in a significant reduction in the ability of the cells to form colonies in soft agar (Supplementary Figure 6B-C). The protein and mRNA expression levels of Ran and Myc were also analyzed in our lung cancer patient cohort and five independent lung cancer datasets available in the GEO database. As shown in Figure 7, increased staining of nuclear Myc protein was significantly associated with nuclear

(Figure 7A and B; spearmen's rank test, $p = 0.002$), cytoplasmic (Figure 7A and C; $p = 0.011$) and overall (Figure 7A and D; $p = 0.001$) staining for Ran protein. Similarly, the level of Ran mRNA was significantly correlated with that of Myc mRNA in all the five independent lung cancer datasets analyzed ($p < 0.05$; Figure 7E-I). Importantly, when the mRNA levels were stratified into four equal groups and the five datasets were combined, a significant correlation between Myc and Ran mRNA levels in lung cancer specimens was also observed ($p < 0.001$; Figure 7J).

The correlation of Ran expression and patient survival in Myc-overexpressing lung cancers Similar to the breast cancer patients, in lung cancer patients with a high immunohistochemical staining for nuclear Myc, a high level of staining for Ran protein was significantly correlated with a shorter survival time (Wilcoxon-Gehan test, $p = 0.048$; Univariate Cox-regression, $\chi^2 = 6.04$, RR = 2.79, 95% CI = 1.23 – 6.31, $p = 0.014$; Figure 8A). In contrast, no significant association between Ran expression and survival was observed in patients with a low level staining for nuclear Myc (Wilcoxon-Gehan test, $p = 0.196$; Figure 8B). When analyzing the combined lung cancer datasets, a high level of Ran expression was significantly correlated with poorer survival (Wilcoxon-Gehan test, $p = 0.001$; Univariate Cox-regression, $\chi^2 = 11.0$, RR = 1.55, 95% CI = 1.12 – 2.00, $p = 0.014$; Supplementary Figure 5B). Moreover, in patients expressing a high level of Myc mRNA, a high level of Ran expression was also associated with a shorter survival time (Wilcoxon-Gehan test, $p = 0.001$; $\chi^2 = 8.17$, RR = 1.99, 95% CI = 1.24 – 3.19, $p = 0.014$; Figure 8C). Again, Ran mRNA expression was not significantly associated with survival in patients with a low level expression of Myc (Wilcoxon-Gehan test, $p = 0.115$; Figure

8D). Together, these results suggest that Ran expression may be an important determinant of Myc-mediated lung cancer progression.

Discussion

Previously, we have shown that Ran silencing results in more potent apoptotic response in cancer cells with activated PI3K/Akt/mTORC1 and Ras/MEK/ERK pathways. In the present study, we investigated the role in Ran in myc-induced tumorigenesis in both breast and lung cancers independent of instant apoptotic response by using a less potent shRNA for Ran knockdown. In this study, we have demonstrated, in immortalized breast epithelial MCF10A cells and breast cancer MDA MB231 cells, that both Ran and Myc *in vitro* are important in enhancing cellular properties, including colony growth in soft-agar, cell adhesion and cell invasion, associated with cancer aggressiveness. When the upstream region of Ran is examined, Myc binds to the distal, but not to the proximal, E-box element and can transactivate the Ran promoter to upregulate Ran expression. The Myc-Ran promoter interaction has also been demonstrated in two other reports using a ChIP-sequencing technique [36, 37]. We have further demonstrated that the *in vitro* observations are of clinical relevance by showing that Myc and Ran protein levels are significantly positively correlated in 288 breast and 102 lung cancer patients, and that their mRNA levels are also significantly positively correlated in 1454 breast and 586 lung cancer patients. *In vitro*, Ran knockdown suppresses the Myc-enhanced colony formation in soft-agar in an immortalized breast epithelial cell line and Ran overexpression reverses this suppression. Most importantly, we have shown that in both breast and lung cancer patients with a high level of Myc expression within the primary tumors, Ran

overexpression is associated with increased death rates. These results provide clinical support to our findings in cultured cells that Ran may be an important downstream effector of Myc-mediated cancer progression.

In the present study, we have also found that Ran is a direct transcriptional target of Myc and that a high level of Ran expression is important for the function of Myc overexpression in breast cancer cells *in vitro* and in Myc overexpressing cancer patients.Myc is overexpressed in more than 40% of human cancers [38], and is, therefore, a good target for cancer therapy. Our results suggest a novel approach for targeting Myc-overexpressing tumors in that silencing of Ran expression may inhibit Myc driven cancer progression. Identification of small molecules that inhibit Ran-RCC1/Ran-RanGAP interactions or that downregulate Ran expression is underway and these molecules could be tested *in vitro* and *in vivo* to validate our hypothesis.

In this study, we have shown that Ran expression is important in Myc overexpressing breast and lung cancers, however, the setting of this study is retrospective and the treatment of the patients, although are in a curative-intent basis, are heterogeneous. Small molecules that inhibit Ran expression or functions are yet to be identified. The investigation of these molecules on inhibiting cancer progression in human patients in a prospective setting is therefore highly warranted.

In our previous [8] report on breast and lung cancers, we have shown that a high level of Ran expression is associated with a shorter survival time in patients particularly those with K-Ras activating mutations, with overexpression of c-Met and osteopontin, with a PIK3CA activating gene signature and in the present report here with Myc

overexpression. These results also suggest that a high level of Ran expression is required for cancers driven by the common oncogenic mutations to express their aggressive behavior. Therefore, Ran may be an important therapeutic target for cancer patients harboring these oncogenic changes. *In vitro* experimental results from us and from others, obtained by silencing Ran, further support the clinical association. Cancer cells are more susceptible to Ran silencing than normal cells [6], while colon cancer cell lines harboring K-Ras activating mutation are more susceptible to Ran silencing than the K-Ras wild-type isogenic counterparts [7, 8]. These data together suggest that Ran is a major downstream target of Myc-overexpressing cancer cells and that Ran may be a novel therapeutic target for cancer cells with oncogenic changes including, but not limited to, Myc overexpression.

Note: The study sponsor did not participate in collection, analysis and interpretation of the data, writing of the manuscript, nor the decision to submit the manuscript for publication.

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Figure Legends

Figure 1. Ran is transcriptionally regulated by Myc

(A) Left panel, Western blot of Myc transfected cells. Stable Myc overexpressing MCF10AT cells had a higher level of Myc (2.1-fold) and a higher endogenous protein level of Ran (2.3-fold) compared to the vector control MCF10AT cells. Right panel, Immunoprecipitation (IP) was performed using anti-RanBP1 antibody, which binds to RanBP1 (binds only to RanGTP), in both MCF10AT-vector and MCF10AT-Myc cells. The resultant pull-down eluents were than analyzed by immunoblotting (IB) for Ran. Actin was used as a loading control for inputs and negative control for the pull-down assay for the two cell lines. (B) Gel electrophoresis of the PCR product from 5'RACE assay showing a specific PCR product of ~200 base pairs. (C) A schematic diagram of Ran promoter. The cysteine residue at +1 position is the transcriptional start site of the *Ran* gene identified by sequencing of the PCR product obtained in (B). (D) Ran promoter activity. Transfection of expression vector for Myc and the Ran promoter reporter construct in MCF10AT cells resulted in a significant increase in the luciferase reporter assay for the Ran promoter activity compared to transfection of control vector and Ran promoter reporter construct (Student's t-test; $p = 0.014$). (E) ChIP assay for the Ran promoter region. Myc binds to the distal E-box element in human Ran promoter *in vivo*. Positive control template was input genomic DNA and negative control template was water. PCR of IgG-pull down ChIP products was used as a control to the Myc-pull down ChIP products for each cell line and are shown side-by-side. (F) A schematic diagram for distal E-box mutagenesis of Ran promoter. Mutations were introduced into the pGL3-RanPro reporter construct. (G) Activities of WT (WTpro) and mutant (Mutpro) Ran promoter. Myc overexpression resulted in a significant increase in relative luciferase activity driven by the Ran promoter in MCF10AT cells (Student's t-test; $p < 0.05$). This Myc-mediated increase in Ran promoter activity was abolished when the sequence of the distal E-box element of the pGL3-Ranpro construct was mutated.

Figure 2. Effect of Myc on in vitro biological properties associated with cancer progression.

(A) Western blots for MCF10A-vector control and MCF10A-Myc cells. MCF10A-vector control and MCF10A-Myc cells were generated by transfection of pBabe-zeo and pBabe-zeo-Myc, respectively, followed by selection in zeocin. Stable overexpression of Myc in MCF10A cells resulted in up-regulation of both Myc (1.7-fold) and the endogenous Ran (2.1-fold). (B) Soft agar assay for MCF10A-vector and MCF10A-Myc cells. Histogram showing mean ± SD for the number of colonies formed in soft agar from three independent experiments. Significantly more colonies grew in soft agar for MCF10A-Myc cells than for MCF10A-vector control cells (Student's t-test). (C) Western blots for MDA MB 231-shScr and MDA MB 231-shMyc cells. MDA MB 231-shScr and MDA MB 231-shMyc cells were generated by retroviral infection of pRetrosuper-shScramble RNA and pRetrosuper-shMyc RNA, respectively, followed by selection in puromycin. Knockdown of Myc by retroviral infection of Myc shRNA resulted in down-regulation of both the endogenous Myc (61% reduction) and the endogenous Ran (30% reduction) protein levels. (D-F) Assays for *in vitro* biological properties, associated with cancer progression and metastasis, of MDA MB 231-shScr and MDA MB 231-shMyc cells. (D) Histogram showing mean ± SD for the number of colonies formed in soft agar from three independent experiments. MDA MB 231-shMyc cells had a significant decrease in number of colonies formed in soft agar assay compared to MDA MB 231-shScr cells (Student's t-test). (E) The same number of MDA MB 231 cells was seeded in the upper chamber of an invasion chamber. They were allowed to migrate through the Matrigel-coated membrane overnight using FBS as chemoattractant. MDA MB 231-shMyc cells had a significant decrease in ability to invade through Matrigel compared to MDA MB 231-shScr cells (Student's t-test). (F) Cells adhering to the fibronectin-coated wells were fixed with 70% ethanol, washed and then stained with crystal violet. The retained crystal violet was extracted and the absorbance was measured. MDA MB 231-shMyc cells had a significant decrease in ability to adhere to fibronectin compared to MDA MB 231-shScr cells (Student's t-test).

Figure 3. Effect of Ran on in vitro biological properties associated with cancer progression and metastasis

(A) Western blots for MCF10A-vector and MCF10A-Ran cells. MCF10A-vector control and MCF10A-Ran cells were generated by retroviral infection of pBabe-hygro and pBabe-hygro-Ran, respectively, followed by selection in hygromycin. A higher protein level of Ran (2.1-fold) was detected in MCF10A-Ran cells compared to MCF10A-vector control cells. (B) Soft agar assay for MCF10A-vector and MCF10A-Ran cells. Bottom panel, Histogram showing mean ± SD for the number of colonies formed in soft agar from three independent experiments. MCF10A-Ran cells had an significant increase in ability to form colonies in soft-agar assay compared to MCF10A-vector control cells (Student's t-test). (C) Western blots for MDA MB 231-shScr and MDA MB 231-shRan cells. MDA MB 231-shScr and MDA MB 231-shRan cells were generated by lentiviral infection of pLKO.1-shScramble RNA and pLKO.1-shRan RNA, respectively, followed by selection in puromycin. MDA MB 231-shRan cells had a lower level of Ran protein at various times after infection (90%, 87% and 83% reduction at 48, 72 and 96 hours, respectively). (D-F) Assays for biological properties associated with cancer progression and metastasis of MDA MB 231-shScr and MDA MB 231-shRan cells. (D) Histogram showing mean ± SD for the number of colonies formed in soft agar from three independent experiments. MDA MB 231-shRan cells formed significantly fewer colonies in soft-agar compared to MDA MB 231-shScr cells (Student's t-test) (E) MDA MB 231-shRan cells had a reduced ability of invading through Matrigel compared to MDA MB 231-shScr cells (Student's t-test). (F) MDA MB 231-shRan cells had a decreased ability to adhere to fibronectin compared to MDA MB 231-shScr cells (Student's t-test).

Figure 4. Effect of Ran on Myc-driven in vitro biological properties associated with cancer progression.

(A) Western blots for MCF10A-vector control-shScr, MCF10A-Myc-shScr and MCF10A-Myc-shRan cells. MCF10A-vector control-shScr, MCF10A-Myc-shScr and MCF10A-Myc-shRan cells were generated by lentiviral infection of pLKO.1-shScr, pLKO.1-shScr and pLKO.1-shRan, respectively, followed by selection in puromycin. MCF10A-Myc-shScr cells had a higher level of endogenous Ran protein (1.6-fold), compared to MCF10A-vector control-shScr cells. This increase in Ran protein level was abolished in MCF10A-Myc-shRan cells upon infection of pLKO.1-shRan construct (87%

reduction). (B) Histogram presenting mean ± SD from three independent experiments for the results from the soft agar assay for MCF10A-vector control-shScr, MCF10A-Myc-shScr and MCF10A-Myc-shRan cells. MCF10A-Myc-shScr cells had an increased ability to form colonies in soft-agar assay. This increase in soft agar colony formation was reduced in MCF10A-Myc-shRan cells (Student's t-test). (C) Western blots for MCF-7-shScr and MCF-7-shRan cells. MCF-7-shScr and MCF-7-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan, respectively, followed by selection in puromycin. Myc, Ran and Actin expression levels for these two cell lines were assessed by Western blot. (D) Histogram presenting mean ± SD from three independent experiments for the results from the soft agar assay for MCF-7-shScr and MCF-7-shRan cells (Student's t-test). (E) Western blots for T47D-shScr and T47D-shRan cells. T47D-shScr and T47D-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan, respectively, followed by selection in puromycin. Myc, Ran and Actin expression levels for these two cell lines were assessed by Western blot. (F) Histogram presenting mean ± SD from three independent experiments for the results from the soft agar assay for T47D-shScr and T47D-shRan cells (Student's t-test).

Figure 5. Association of protein and mRNA levels of Myc and Ran in breast cancer specimens.

(A-F) Representative images of immunohistochemical staining of breast cancers for (A, C, E) Myc and separately for (B, D ,F) Ran. Two separate histological sections from each of 3 tumors are shown: tumor 1 (A and B), tumor 2 (C and D), tumor 3 (E and F). Sections were immunohistochemically stained for Myc in A,C,E and for Ran in B,D,F. In tumor 1, carcinoma cells (c) were unstained for (A) Myc and for (B) Ran, whilst reactive stromal cells (arrows) were well stained with either antibody. In tumor 2, nuclei of carcinoma cells were well stained for (C) Myc (arrows) and for (D) Ran (arrows), there was little or no staining of carcinoma cell cytoplasm with either antibody. In tumor 3, (E), the cytoplasm and some nuclei (arrows) of the carcinoma cells were stained for Myc, whilst (F) only the cytoplasm was stained for Ran.Magnification A-D 390x, E and F 960x. Bars A-D, 25 μm; E and F, 10 μm. (G,H) comparison of immunohistochemical staining

in all the 288 different tumors for (G) staining for nuclear Myc and for nuclear Ran, and (H) staining for nuclear Myc and for cytoplasmic Ran. Fisher's exact test was used (I-P) Comparison of relative levels of Myc and Ran mRNA in breast cancer datasets available in the GEO database. Relative mRNA levels of Myc and Ran was extracted from the GEO database and normalized by using R and Bioconductor as previous described [8]. Datasets with patient number more than or equal to 150 were included and the correlations between relative mRNA levels of Myc and Ran in each dataset were compared by non-parametric bivariate correlation analysis (Spearman's rank test) using SPSS. (I-O) In all the datasets tested in the present study, (I) GSE1456 (n = 159), (J) GSE2034 (n = 286), (K) GSE3143 (n = 158), (L) GSE4922 (n = 255), (M) GSE7390 (n = 198), (N) GSE1121 (n = 200) and (O) GSE12276 (n = 204), the relative mRNA levels of Myc and Ran were significantly positively correlated (Spearman's rank test; $p < 0.05$). (P) Histogram showing percentage of cases with different levels of Ran mRNA in patients with different levels of Myc mRNA. The 7 datasets were combined into one by stratifying the mRNA levels of Myc and Ran into four groups equally using the three quartiles as cut-off points. In the combined breast cancer dataset (n = 1454), the association between mRNA levels of Myc and Ran was tested by Spearman's rank test. Significantly more patients with a higher level of Myc mRNA also had a higher level of Ran mRNA (Chi-square test; $p < 0.001$).

Figure 6. Association of expression of Myc and Ran alone and in combination with patient survival in breast cancer

(A-F) Kaplan-Meier plots of the cumulative proportion of patients surviving with time in months of the Liverpool breast cancer patient cohort (n = 288) are shown for (A) tumors with nuclear Myc alone, (B) tumors stained for cytoplasmic Myc alone, (C) nuclear Myc positive tumors with either positive or negative staining for nuclear Ran, (D) nuclear Myc negative tumors with either positive or negative staining for nuclear Ran, (E) nuclear Myc positive tumors stained for either positive or negative staining for cytoplasmic Ran and, (F) nuclear Myc negative tumors stained for either positive or negative staining for cytoplasmic Ran, and (G – H) Kaplan-Meier plots of cumulative proportion of patients surviving with time in years of the combined breast cancer dataset (n = 1454) from the

GEO database for mRNA level of Ran in patients with (G) a high level and (H) a low level of Myc mRNA expression. Wilcoxon-Gehan test was used.

Figure 7. Association of expression of Myc and Ran in lung cancer patients.

(A) Representative images of immunohistochemical staining for Myc and Ran in lung cancer specimens. (B – D) Quantitative comparison of staining for Myc and Ran in different tumors for (B) staining for nuclear c-Myc and for nuclear Ran, (C) staining for nuclear c-Myc and for cytoplasmic Ran, and (D) staining for nuclear c-Myc and overall Ran. Fisher's exact test was used in B and C, and Chi-square test was used in D. (E – J) Comparison of relative levels of Myc and Ran mRNA in lung cancer datasets available from the GEO database. (E – I) Relative mRNA levels of Myc and Ran was extracted from the GEO database and normalized by using R and Bioconductor as previously described [8]. Datasets with patient number more than or equal to 90 were included and the correlations between relative mRNA levels of Myc and Ran in each dataset were compared by non-parametric bivariate correlation analysis (Spearman's rank test) using SPSS. In all the datasets tested in the present study, (E) GSE3141 (n = 111), (F) GSE4573 (n = 130), (G) GSE8894 (n = 138), (H) GSE13213 (n = 117) and (I) GSE14814 (n = 90), the relative mRNA levels of Myc and Ran were significantly positively correlated (Spearman's rank test; $p < 0.05$). Note that the values for Myc and Ran in the GSE13213 dataset were negative because the microarray was performed using a two channel method were the reference sample was RNA extracted from 20 lung cancer cell lines, the results indicated that most of the human specimens had a lower expression of Myc and Ran compared to the reference sample (20 lung cancer cell lines). (J) The 5 datasets were combined into one by stratifying the mRNA levels of Myc and Ran into four groups equally using the three quartiles as cut-off points. In the combined lung cancer dataset (n = 586), the association between mRNA levels of Myc and Ran was tested by Spearman's rank test. Significantly more patients with a higher level of Myc mRNA also had a higher level of Ran mRNA (Chi-square test; $p < 0.001$).

Figure 8. Association of expression of Myc and Ran alone and in combination in lung cancer patients.

(A - B) Kaplan-Meier plots of the cumulative proportion of patients surviving with time in months of the Dublin lung cancer patient cohort are shown for tumors stained for (A) low and (B) high level of nuclear Myc for high, intermediate and low levels of overall (cytoplasmic and nuclear) Ran protein expression. (C – D) Kaplan-Meier plots of the cumulative proportion of patients surviving with time in months of the combined lung cancer dataset (n = 586) from GEO database for mRNA level of Ran in patients with (C) a low and (D) a high level of Myc mRNA. Wilcoxon-Gehan test was used.

Supplementary Figure 1. Schematic diagram for the human Ran promoter.
Transcriptional start site of Ran was defined by 5'RACE as the cytosine at the +1 position in the schematic diagram. The 718 base-pair upstream sequence of the human Ran promoter, containing the two E-box elements was cloned into the reporter luciferase construct, pGL-3, for luciferase assay. The DNA sequence of this promoter region was analyzed *in silico* using genomatix software for prediction of potential binding sites of different transcription factors.

Supplementary Figure 2. Schematic diagram showing gene specific primers for 5'RACE and Ran mRNA sequence, the large C marks the transcriptional start site.

Supplementary Figure 3. Effect of knockdown of Ran using shRan1 and shRan4 RNA.
MDA MB 231 cells were transiently infected with lentivirus containing the shRan1 and shRan4 and control scrambled shRNA (shScr) for different times and the resultant cells were analyzed for Ran and actin proteins by Western blot. shRan4 was not as potent as shRan1 for Ran knockdown in terms of the reduction of Ran protein expression as well as induction of apoptosis as measured by % subG1 cells. These results are corroborate those in our previous publication using 5 different shRNAs targeting Ran [8].

Supplementary Figure 4. Effect of Ran overexpression or knockdown on Myc expression in breast cancer cell lines. (A) Overexpression of Ran in MCF10AT cells resulted in an up-regulation of Ran expression but did not change the expression of Myc. (B)

Knockdown of Ran in MDA MB231 cells resulted in a reduction in Ran expression but did not change the expression of Myc. Actin was used as a loading control.

Supplementary Figure 5. The Kaplan-Meier plots for Ran in breast and lung cancers. (A) Survival data and Ran mRNA expression from seven independent breast cancer patient datasets available in the GEO and Prognoscan database (n = 1454) were combined and the Kaplan-Meier plot for Ran in this combined dataset was shown. (B) The survival data and Ran mRNA expression from five independent lung cancer patient datasets available in the GEO and Prognoscan database (n = 586) were combined and the Kaplan-Meier plot for Ran in this combined dataset was shown. P-value, by Wilcoxon-Gehen test, was shown alongside the plot. In the previous study, we have included only 266 breast and 296 lung cancer patients, while in this study, we have included 1454 breast and 586 lung cancer patients in our survival and correlation analyses. Wilcoxon-Gehan test was used.

Supplementary Figure 6. Effect of Myc overexpression on Ran promoter activity and the effect of Ran knockdown on colony formation in soft agar in A549 lung cancer cells (A) Co-transfection of Myc overexpressing vector and pGL3-Ranpro reporter construct resulted in higher luciferase activity in A549 cells than co-transfection of control vector and pGL3-Ranpro. Luciferase activity was normalized with Renilla luciferase as a internal control in the dual luciferase assay (Student's t-test). (B) Western blots for A549-shScr and A549-shRan cells. A549-shScr and A549-shRan cells were generated by lentiviral infection of pLKO.1-shScr and pLKO.1-shRan, respectively, followed by selection in puromycin. Myc, Ran and Actin expression levels for these two cell lines were assessed by Western blot. (C) Histogram presenting mean ± SD from three independent experiments for the results from the soft agar assay for A549-shScr and A549-shRan cells (Student's t-test).