RRM1 domain of the splicing oncoprotein SRSF1 is required for MEK1-MAPK-ERK activation and cellular transformation

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Alternative splicing regulators have emerged as new players in cancer development, modulating the activities of many tumor suppressors and oncogenes and regulating the signaling pathways. However, little is known about the mechanisms by which these oncogenic splicing factors lead to cellular transformation. We have shown previously that the splicing factor serine and arginine splicing factor 1 (SRSF1; SF2/ASF) is a proto-oncogene, which is amplified in breast cancer and transforms immortal cells when overexpressed. In this study, we performed a structure–function analysis of SRSF1 and found that the RNA recognition motif 1 (RRM1) domain is required for its oncogenic activity. Deletion of RRM1 eliminated the splicing activity of SRSF1 on some of its endogenous targets. Moreover, we found that SRSF1 elevates the expression of B-Raf and activates the mitogen-activated protein kinase kinase (MEK) extracellular signal-regulated kinase (ERK) pathway and that RRM1 is required for this activation as well. B-Raf-MEK-ERK activation by SRSF1 contributes to transformation as pharmacological inhibition of MEK1 inhibits SRSF1-mediated transformation. In conclusion, RRM1 of SRSF1 is both required (and when tethered to the RS domain) also sufficient to activate the Raf-MEK-ERK pathway and to promote cellular transformation.

Introduction

Many oncogenes and tumor suppressors are differentially spliced in cancer cells, and it has been shown that many of these cancer-specific isoforms contribute to the transformed phenotype of cancer cells (1–3). Moreover, mutations in components of the splicingosome were recently discovered in several cancers and are predicted to be driver mutations supporting the concept that splicing factors are important players in cancer development (4–6). However, only recently, evidence has shown direct contribution of alternative splicing regulators to cancer development and progression (7–9).

Serine and arginine (SR) proteins are a family of abundant RNA-binding proteins with multiple roles in constitutive and alternative splicing, as well as additional roles in other aspects of gene expression (10–13). Members of this family have closely related sequences and a modular structure with one or two copies of a RNA recognition motif and a C-terminal arginine/serine-rich (RS) domain that undergoes reversible phosphorylation (10–13). Their general splicing function was revealed by the requirement of at least one family member for spliceosome assembly and splicing of any pre-messenger RNA (mRNA) in vitro (14). Involvement of SR proteins in alternative splicing and exon definition is evident from their pronounced concentration-dependent effects on alternative splice-site selection in vitro and in cells, and from their ability to enhance splicing via cognate exonic splicing enhancer elements (15). Additional effects of particular SR proteins on mRNA export, non-sense-mediated decay (16) and translation were reported previously (17), and in some cases reflect the ability of some SR proteins to exit the nucleus bound to mRNA and then shuttle back to the nucleus (18). SR proteins are ubiquitously expressed, but at levels that are characteristic of each cell type (8). Individual SR proteins are essential at the organismal and cellular levels, and reduced expression of one family member was found to elicit genomic instability and apoptosis (19,20).

We have shown previously that the splicing factor serine and arginine splicing factor 1 (SRSF1; SF2/ASF) is overexpressed in several cancers, its gene is amplified in breast tumors and its overexpression induces transformation and tumorigenicity (8,21). Moreover, we and others have identified several tumor suppressors and oncogenes regulated by SRSF1 (8,21,22). Previous studies have examined the contribution of SRSF1 structural domains to its splicing activity and RNA binding in vitro or using transient transfections (23–27). However, the contribution of SRSF1 domains to cellular transformation and alternative splicing effects in vivo were not studied thoroughly. Here, we examined the contribution of SRSF1 structural domains to cellular transformation and regulation of its splicing targets in an immortal liver-progenitor xenograft model (28) and other cellular systems. We found that although RNA recognition motif 2 (RRM2) is dispensable for transformation and activation of some splicing targets, RNA recognition motif 1 (RRM1) domain is both required, and when tethered to the RS domain, also sufficient to activate the Raf-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway and to promote cellular transformation.

Materials and methods

Cells

Liver progenitor cells from embryonic day 18 p53−/− fetal livers were isolated and immortalized with MSCV-based retroviruses expressing Myc-ires-GFP as described (28) to generate p53−/− hepatocytes–Myc (PHM-1) cells. PHM-1, BEAS-2B (human immortal lung bronchial epithelial cells) and HEK 293T (human embryonic kidney expressing SV40 T antigen) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal calf serum, penicillin and streptomycin. To generate stable cell pools, PHM-1 cells were infected with pWZL-hydro retroviral vector expressing T7-tagged SRSF1 mutant complementary DNAs. BEAS-2B cells were infected with a pBABE-puro retroviral vector expressing T7-tagged SRSF1 complementary DNA. We replaced the medium 24 h after infection, and 24 h later, infected cells were selected with hygromycin (200 μg/ml) for 96 h or with puromycin (2 μg/ml) for 72 h. HEK 293T cells were transiently transfected using Fugene 6 (Roche Diagnostics) according to manufacturer’s recommendations.

Growth curves

PHM-1 cells were infected with retroviruses expressing T7-tagged SRSF1 mutant complementary DNAs. After selection, 2000 cells per well were seeded in 96-well plates. Cells were fixed and stained with methylene blue as described previously (8), and the A560 of the acid-extracted stain was measured on a plate reader (Bio-Rad).

Xenograft tumor formation in nude mice

PHM-1 stable pools expressing SRSF1 mutants were injected (3 × 10⁶ cells per site in 200 μl of phosphate-buffered saline) subcutaneously into each rear flank

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SRSF1 activation of the Ras-MAPK-ERK pathway requires RRM1.

**Immunoblotting**

Cells were lysed in Laemmli buffer and analyzed for total protein concentration as described previously (9). Twenty to fifty micrograms of total protein from each cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked and probed with antibodies, using enhanced chemiluminescence detection. Primary antibodies were β-catenin (1:2000; Sigma), actin (1:2000; Santa Cruz Biotechnology), glyceraldehyde 3-phosphate dehydrogenase (1:1000; Santa Cruz), SRSF1 (mAb AK96 culture supernatant, 1:200; 18), T7 tag (1:5000; Novagen), BRAF (1:1000; Santa Cruz Biotechnology), phospho-ERK1/2 (1:1000, Sigma), ERK1/2 (1:1000; Cell Signaling), phospho-MEK1/2 (1:1000; Cell Signaling), MEK1/2 (1:1000; Cell Signaling), phospho-AKT (Cell Signaling) and AKT (Cell Signaling). Secondary antibodies were horseradish peroxidase-conjugated goat, anti-mouse or anti-rabbit IgG (1:10 000; Jackson Laboratories).

**Reverse transcriptase-Polymerase Chain Reaction**

Total RNA was extracted with Tri reagent (Sigma) and 1 µg of total RNA was reverse transcribed with M-MLV (Promega). PCR was performed in 50 µl reactions containing 0.2 µM of deoxynucleoside triphosphate mix, 1× PCR buffer with 15 mM of MgCl₂, 1 unit of Super-Therm GOLD (Bertec Enterprise), 5 pM of each primer and 8% (vol/vol) dimethyl sulfoxide. PCR conditions were 95°C for 10 min, then 95°C for 30 s, 60°C for 30 s and 72°C for 45 s for 30–35 cycles, followed by 10 min at 72°C. PCR products were separated on 1.5% agarose gels or on 6% non-denaturing polyacrylamide gels. Primer sequences are listed in Supplementary Table S1, available at Carcinogenesis Online.

**Quantitative reverse transcriptase–Polymerase Chain Reaction**

Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed using SYBR Green (SYBR Premix Ex Taq # RR041A) and the CFX96 machine (Bio-Rad). qRT–PCR conditions were 95°C for 10 min, then 39 cycles of 95°C for 10 s, 58°C for 20 s, followed by 10 s at 95°C, 5 s at 65°C and 0.5 s at 95°C.

**Anchorage-independent growth**

Colonies in soft agar were assayed as described previously (8). Plates were incubated at 37°C and 5% CO₂. After 14 days, colonies from 10 different fields in each of two wells were counted for each transfectant pool, and the average number of colonies per well was calculated. U0126 inhibitor (LC Laboratories) was used at a final concentration of 25 µM.

**Results**

**Oncogenic activities of SRSF1 deletion mutants**

The splicing factor SRSF1 is composed of a classical conserved RRM1, a short linker domain, a second pseudo RRM2 and a RS domain (Supplementary Figure S1, available at Carcinogenesis Online). Previous in vitro studies showed that RRM1 is dispensable for RNA binding, in vitro splicing and non-sense-mediated decay activities of SRSF1 (16,24,25,29). However, the effects of SRSF1 mutants on endogenous targets showed different results (21). The contribution of SRSF1 structural domains to cellular transformation has not been studied yet. We investigated the mechanisms of SRSF1-mediated transformation through a structure–function analysis of its specific domains. We examined the oncogenic activities of a series of SRSF1 mutants; ΔRRM1, ΔRRM2 and ARS mutants are deleted in the RRM1, RRM2 and RS domains, respectively (24,25,29). The NRS1 mutant is a nuclear retention mutant that contains the RS domain of the SR protein SRSF2 (SC35), which is unable to exit the nucleus or shuttle between the nucleus and the cytoplasm (29,30; Supplementary Figure S1, available at Carcinogenesis Online). In order to determine the oncogenic activities of these mutants, we tested whether their overexpression can transform immortal liver-progenitor cells (PHM-1), which upon transduction with oncogenes form hepatocellular carcinoma tumors (28). We transduced pools of PHM-1 cells with retroviruses expressing T7-tagged SRSF1 deletion mutants (Supplementary Figure S1, available at Carcinogenesis Online; Figure 1A). To assess whether transforming activity of the mutants was due to their ability to increase cell proliferation, we measured the proliferation rates of PHM-1 transduced cells. We found that all the cell pools proliferated similarly to control cells, indicating that the mutants did not affect cell proliferation (Figure 1B). Cells expressing the different mutants were injected into nude mice. Cells overexpressing either wild-type-SRSF1 (WT-SRSF1) or ΔRRM2 or ΔRS mutants gave rise to large tumors in a similar manner. Overexpression of ΔRRM1 and NRS1 mutants resulted in the formation of small tumors or no tumors at all (Figure 1C and D).

**The effects of SRSF1 deletion mutants on alternative splicing of endogenous targets**

As mentioned previously, in vitro splicing assays and co-transfection of minigenes together with SRSF1 deletion mutants have suggested that RRM1 is dispensable for SRSF1’s alternative splicing activity, which is thought to be mediated mainly by RRM2 (16,24,25,30). However, examination of endogenous alternative splicing events after stable overexpression of SRSF1 deletion mutants showed different results; alternative splicing of several endogenous targets was affected by RRM1 but not by RRM2 (21). Thus, we investigated the effects of SRSF1 mutants on alternative splicing of some well-characterized endogenous targets of SRSF1, either by transient overexpression or by stable transduction. Specifically, we examined the splicing patterns of the tumor-suppressor BIM, the transcription factor TEAD-1 (TEF-1) and the pro-apoptotic Bcl-2 family member BIM (31–33). The BIM protein interacts with the c-Myc oncoprotein to suppress its oncogenic activity. The inclusion of exon 12A abolishes the tumor-suppressor activity of BIM, whereas the inclusion of exon 13 is required for BIM’s tumor suppressor activity (32). It was shown previously that SRSF1 overexpression in human, mouse and rat cells promotes the inclusion of exon 12A (8,21). We measured the relative inclusion of exon 12A in tumors formed in nude mice injected with PHM-1 cells stably transduced with SRSF1 deletion mutants. We found that in tumors derived from cells overexpressing WT-SRSF1, the inclusion of exon 12A was increased compared with the empty vector control (Figure 2A). ARS and NRS1 mutants promoted a similar increase in inclusion of exon 12A but to a smaller extent than WT-SRSF1. Notably, ΔRRM2 mutant induced a sharp increase in 12A/13 inclusion ratio, while ΔRRM1 behaved similarly to the empty vector control (Figure 2A). SRSF1 also affects the alternative splicing of the pre-mRNAs of the transcription factor TEAD-1 (TEF-1) by promoting the inclusion of exon 5 (8). Consistent with this, we found increased inclusion of exon 5 of TEAD-1 in tumors derived from SRSF1-overexpressing cells (Figure 2A). Overexpression of ΔRRM2 induced a similar increase, whereas tumors derived from ΔRRM1-overexpressing cells had a similar splicing pattern as empty vector control. ARS mutant promoted a slight increase in inclusion of exon 5, and surprisingly, NRS1 did not induce any increase in exon 5 inclusion. Finally, we examined the alternative splicing of the pro-apoptotic Bcl-2 family member BIM transcripts. It was shown recently that overexpression of SRSF1 in the non-transformed human breast cells MCF-10A inhibits apoptosis by generating a new isoform of BIM, named γ1 (21). This variant lacks the region encoding the BH3 domain, which is necessary for induction of apoptosis by BIM (21). Overexpression of γ1 in MCF-10A cells grown in matrigel induces an increase in acinar size and a decrease in apoptosis (21). We found, in tumors derived from overexpression of SRSF1 and ΔRRM2 in immortal mouse liver progenitors, inclusion of a new alternative exon (we named exon 3a), instead of exon 4 (Figure 2A and Supplementary Figure S2, available at Carcinogenesis Online). To keep the nomenclature of BIM isoforms consistent, we named this isoform BIM extra-short. This transcript does not contain exon 4, which encodes the BH3 domain responsible for pro-apoptotic activity of BIM, so its pro-apoptotic activity is suppressed similar to the structure of the human BIM γ1 (21; Supplementary Figure S2, available at Carcinogenesis Online). In order to confirm the effects of SRSF1 mutants on alternative splicing of endogenous targets, we transfected HEK 293T cells with these mutants. We transiently overexpressed T7-tagged version of SRSF1 deletion mutants in HEK 293T cells (Figure 2B). In the case of BIM, we found similar results as in PHM-1 derived tumors. Cells overexpressing WT-SRSF1 and ΔRRM2 showed increased inclusion of exon 12A and
reduced inclusion of exon 13, whereas ΔRRM1-overexpressing cells behaved similarly to the control cells. Both ΔRS and NRS1 mutants promoted increased inclusion of exon 12A and corresponding reduced inclusion of exon 13, but to a smaller extent than WT-SRSF1. We also examined alternative splicing of the pro-apoptotic Bcl-2 family member BIM transcripts. Similar to previous reports (21), we observed a significant increase in γ1 level in HEK 293T cells overexpressing SRSF1 (Figure 2B). Cells overexpressing the ΔRS mutant had comparable levels of splicing as cells overexpressing WT-SRSF1, whereas cells overexpressing NRS1 mutant promoted a lower level of γ1. Finally, ΔRRM1- and ΔRRM2-overexpressing cells displayed γ1 levels similar to control cells (empty vector; Figure 2B).

Effects of SRSF1 mutants on mitogen-activated protein kinase signaling

The Ras-mitogen-activated protein kinase (MAPK) pathway is deregulated in many cancers including hepatocellular carcinoma and contributes to cancer development and progression (34,35). Thus, we investigated the effects of SRSF1 mutants on ERK1 and ERK2 activation in PHM-1 cells. We found a significant increase in ERK1/2 phosphorylation in cells that overexpressed WT-SRSF1, ΔRRM2 and ΔRS mutants (Figure 3A). Furthermore, the upstream kinases, MEK1 and MEK2, which are responsible for ERK1 and ERK2 phosphorylation, were activated to a similar extent as measured by phosphorylation (36,37; Figure 3A). In contrast, there was no increase in MEK1-ERK1/2 activation in ΔRRM1- and NRS1-overexpressing cells (Figure 3A). These results demonstrate a good correlation between mutants that activate the MAPK-ERK pathway and their ability to induce tumorigenesis because only SRSF1 mutants that induced tumor formation in nude mice showed MEK1-ERK1/2 activation (Figures 1C, D and 3A). The phosphatidylinositide 3-kinase–Akt pathway, another important signaling pathway that is activated in many cancers (38), was not activated by SRSF1 or by any of the SRSF1 mutants (Figure 3A). We further examined the expression of B-RAF at the RNA and protein levels in tumors derived from PHM-1 cells that overexpressed WT-SRSF1, ΔRRM2 and ΔRS. qRT-PCR analysis showed that B-RAF transcripts were elevated compared with tumors derived from cells containing empty vector, 2-, 4- and 12-fold, respectively (Figure 3B). B-RAF protein levels were also significantly increased in tumors expressing WT-SRSF1, ΔRRM2 and ΔRS compared with tumors derived from control cells, with the greatest increase in tumors derived from ΔRRM2-overexpressing cells (Figure 3C). Given the correlation between the ability of SRSF1 mutants to activate the MAPK-ERK pathway and their ability to induce tumorigenesis, we sought to determine whether MEK-ERK activation is required for SRSF1-mediated transformation. Thus, we treated PHM-1 cells transformed by WT-SRSF1, ΔRRM2 or ΔRS with the MEK1 inhibitor U0126 (39). We found that colony formation in soft agar of PHM-1 cell pools transduced with WT-SRSF1, ΔRRM2 or ΔRS was reduced 7-
SRSF1 activation of the Ras-MAPK-ERK pathway requires RRM1

5- and 4-fold, respectively, in the presence of the MEK1 inhibitor U0126 compared with vehicle. This suggests that transformation by SRSF1 or SRSF1 mutants requires MEK-ERK activation (Figure 4A). Activation of the MEK-ERK pathway and sensitivity of SRSF1 transformed cells to MEK1 inhibition was also demonstrated in BEAS-2B overexpressing SRSF1 (Figure 4B and C).
A.Shimoni-Sebag et al.

Discussion

The splicing factor oncoprotein SRSF1 has been shown to be a potent proto-oncogene that is upregulated in many cancers and can transform immortal mouse and human cells (8,21). SRSF1 can also induce senescence in normal cells by stabilization of TP53 through protein–protein interactions and independently of RNA binding (40). SRSF1 is involved in the regulation of several mRNA processing steps such as mRNA transport, splicing, alternative splicing, non-sense-mediated decay, DNA–RNA R-loop formation, translation and micro RNA biogenesis (16,17,41–43). Thus, it is not clear which of these activities is required for SRSF1 oncogenic activity and which structural domain of SRSF1 mediates this activity. Here, we sought to characterize the domains of SRSF1 required for its activity as an oncoprotein.

RRM1 of SRSF1 is required for cellular transformation

We found that the RRM1 domain was required for the oncogenic activity of SRSF1, whereas the RRM2 domain was dispensable for this activity. The RS domain was also dispensable as the ARS mutant could induce tumor formation (Figure 1). Surprisingly, the NRS1 mutant harboring the SRSF2 (SC35) RS domain, which is constantly nuclear (29,30), was unable to induce tumors (Figure 1), suggesting that the shuttling activity of SRSF1 is required for its oncogenic activity.

RRM1 of SRSF1 is required for alternative splicing regulation

In contrast with in vitro splicing studies and transient co-transfection assays using minigenes, in our study the mutant containing only the RRM1 domain tethered to the RS domain of SRSF1 was sufficient to affect alternative splicing of some endogenous splicing targets of SRSF1 such as BIM, BIM and TEAD1 (Figure 2). Interestingly, some splicing events, such as BIM exon γ1 inclusion were affected by WT-SRSF1, NRS1 and the ARS mutants, but not by the ARRM2 mutant, suggesting that either some of the effects of the ΔRRM2 mutant are indirect, or that it possesses some different specificity toward some of the splicing targets. Another possibility is that natural chromatin environment is required for proper alternative splicing regulation in this case. The NRS1 mutant, even though possessing all the structural domains of SRSF1, was unable to affect the splicing of some endogenous targets such as mouse BIM and TEAD1, correlating with its inability to induce tumorigenesis (Figures 1 and 2). This result suggests that the addition of the SRSF2 RS domain might change the specificity of SRSF1 toward its splicing targets. Previous in vitro splicing assays have demonstrated that SRSF1 RRM1 and RRM2 but not its RS domain dictate its substrate specificity, and replacement of the SRSF1 RS domain with the SRSF2 RS domain did not affect its substrate specificity (26,27). A possible explanation for this discrepancy is that in vivo, the SRSF2 RS domain might affect the nuclear localization of SRSF1 (SRSF2 might be localized to speckles in a different manner than SRSF1). Another possibility is that the SRSF2 RS domain is differentially phosphorylated or interacts differentially with other proteins in vivo (30,44). A recent study found similar differences in the alternative splicing activities of SRSF1 structural mutants, corroborating our findings (21).

A genome-wide study shows that indeed although in some cases, SRSF1 and SRSF2 splicing activity is redundant, in other cases, they have antagonistic effects on splicing of some splicing targets (45).

RRM1 of SRSF1 is required for Raf-MEK-ERK activation

In order to better understand the oncogenic properties of SRSF1, we examined the activity of the Ras-Raf-MAPK pathway in cells overexpressing the different mutants. The Ras-MAPK pathway is hyperactivated in many tumors, and many of its components are activated by mutations in tumors including Ras (34,46), Raf (47) and MEK1 (35), as well as upstream activators such as the epidermal growth factor receptor (48). We found that SRSF1 activated this pathway as measured by ERK1/2 and MEK1 phosphorylation (Figure 3). In correlation with their tumorigenic activity, the ΔRRM2 and ΔRS mutants activated MEK1-ERK, whereas ARRM2 and the NRS1 mutants did

Fig. 4. Inhibition of MAPK-ERK signaling blocks SRSF1-, ΔRRM2- and ΔRS-mediated transformation. (A) Quantification of soft agar colony formation. PHM-1 cells stably overexpressing SRSF1, ΔRRM2 or ΔRS were seeded in soft agar with or without the MEK1 inhibitor UO126 and colonies were counted 14 days later. The mean ± SD for each pair of pooled cell lines is shown. (B) BEAS-2B cells were stably transduced with retroviruses expressing empty vector or T7-tagged SRSF1 and total protein was isolated. Western blot was carried out using the indicated primary antibodies. (C) BEAS-2B cells transduced with empty vector or SRSF1 were seeded into soft agar with or without the MEK1 inhibitor UO126 and colonies were counted 14 days later. Mean ± SD (n = 2) are shown.
not (Figure 3). These results suggest that RRM1 is required and sufficient to induce activation of this signaling pathway. Furthermore, we found that WT-SRSF1, ARRM2 and ΔRRS induced elevation of B-RAF mRNA and protein levels in tumors derived from overexpression of these mutants in PHM-1 cells (Figure 3B and C). B-RAF upregulation might explain the activation of MEK1-ERK1/2 as B-RAF is one of its upstream kinases together with A- and C-Raf (36,37). The mechanism by which B-RAF is upregulated upon SRSF1 overexpression is unknown and requires further investigation. Moreover, it is possible that other components of the Ras-Raf-MAPK are affected by SRSF1 either by its alternative splicing activity or other activities. Finally, in order to understand if activation of MEK-ERK is important for SRSF1-mediated transformation, we treated cells transformed by WT-SRSF1, ARRM2 or ΔRRS with the MEK1 inhibitor U0126. We found that cells transformed by SRSF1 or ARRM2 and ΔRRS were sensitive to MEK1 inhibition and did not form colonies in soft agar in the presence of the inhibitor (Figure 4). These results indicate that activation of MEK1-ERK1/2 is required for SRSF1-mediated transformation. SRSF1 is a potent oncogene. This study unveils the essential role of SRSF1’s RRM1 domain for splicing regulation, MAPK activation and cellular transformation. Moreover, we identified a possible mode by which SRSF1 leads to cellular transformation and activation of the Ras-MAPK pathway. These findings suggest that tumors overexpressing SRSF1 might be sensitive to Raf/MEK/ERK inhibitors, while being insensitive to upstream kinase inhibitors such as epidermal growth factor receptor inhibitors, due to the possibility that SRSF1 may activate the Ras-Raf-MEK-ERK pathway downstream by elevating B-RAF levels. A possible efficient and specific drug therapy would be to target directly the RRM1 of SRSF1, rather than to inhibit indirectly the Ras-Raf-MAPK pathway downstream by elevating B-RAF or oligonucleotide that will compete, or interfere, with the binding of SRSF1 RRM1 to its target. Recent attempts, mainly by the Tazi group, to develop a small molecule that will interfere with SRSF1 splicing activity shows promising results in human immunodeficiency virus infection and might be developed to include cancer treatment (49–52).

Supplementary material
Supplementary Table S1 and Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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References


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