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Glypican-5 is a tumor suppressor in non-small cell lung cancer cells

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ABSTRACT

Glypican-5 (GPC5) belongs to the glypican family of proteoglycans that have been implicated in a variety of physiological processes, ranging from cell proliferation to morphogenesis. However, the role of GPC5 in human cancer remains poorly understood. We report that knockdown of GPC5 in bronchial epithelial cells promoted, and forced expression of GPC5 in non-small lung cancer (NSCLC) cells suppressed, the anchorage-independent cell growth. *In vivo*, expression of GPC5 inhibited xenograft tumor growth of NSCLC cells. Furthermore, we found that GPC5 was expressed predominantly as a membrane protein, and its expression led to diminished phosphorylation of several oncogenic receptor tyrosine kinases, including the ERBB family members ERBB2 and ERBB3, which play critical roles in lung tumorigenesis. Collectively, our results suggest that GPC5 may act as a tumor suppressor, and reagents that activate GPC5 may be useful for treating NSCLC.

1. Introduction

Glypicans are a family of multifunctional proteoglycans and widely expressed in both invertebrates and vertebrates as glycophosphatidylinositol (GPI)-anchored membrane-bound proteins [1–3]. In drosophila, mutation of the glypican gene Dally (division abnormally delayed) led to cell division defects that severely impaired developmental morphogenesis in adult tissues, such as the eye and the wing [4]. In mammalian cells, six glypicans (GPC1-6) have been identified. GPC5 was originally isolated as a brain-enriched gene and then found to be developmentally regulated in various organs and tissues, including the central nervous system, the limb, and the kidney [5,6]. Interestingly, GPC5 gene is located to the 13q31-32 chromosomal region that is frequently mutated or amplified in human diseases, including cancer [7–9], suggesting a candidate role for GPC5 in regulating carcinogenesis.

Lung cancer has become the leading cause of cancer-related deaths among all human malignancies. Despite our continuous technical improvements, the five-year survival rate of lung cancer has not significantly increased during the past decades, partly due to our incomplete understanding of the biologic processes that regulate lung tumorigenesis. Notably, several recent studies have shown that single-nucleotide polymorphisms (SNPs) of GPC5 associated with the risk of lung cancer [10–13], and the expression level of GPC5 was decreased in lung adenocarcinomas compared to normal lung tissues [10,14], implying that GPC5 may be a tumor suppressor. However, *in vitro* studies have generated controversial results about the role of GPC5 in lung cancer. For instance, one recent report has shown that higher GPC5 expression was associated with lymph node metastasis and poorer prognosis of non-small cell lung cancer (NSCLC) patients; and overexpression of GPC5 promoted cell migration [15], suggesting a pro-oncogenic role for GPC5 in NSCLC. In a sharp contrast, another study showed that lower GPC5 was associated with lymph node metastasis and predicted shorter survival of NSCLC patients, and overexpression of GPC5 in NSCLC cells induced cell cycle arrest and inhibited migration and invasion *in vitro*, suggesting a metastasis suppressor role for GPC5 [16]. Despite these results, the *in vivo* role of GPC5 remains untested, and mediators of GPC5 are unclear.

To address these issues, we knocked down GPC5 in bronchial epithelial cells and overexpressed it in non-small cell lung cancer cells. We found that knockdown of GPC5 promoted, and overexpression of GPC5 inhibited, the anchorage-independent cell growth in soft agar, suggesting that GPC5 suppresses the tumorigenicity of these cells. Most importantly, overexpression of GPC5 significantly inhibited the growth of xenograft tumors formed by lung adenocarcinoma cells, indicating that GPC5 acts as a tumor suppressor *in vivo*. Furthermore, we provide evidence that GPC5 is localized exclusively to the cellular membrane, where it may repress several oncogenic receptor tyrosine kinases, including RYK, ERBB2, and ERBB3, to exert its tumor suppressive function.

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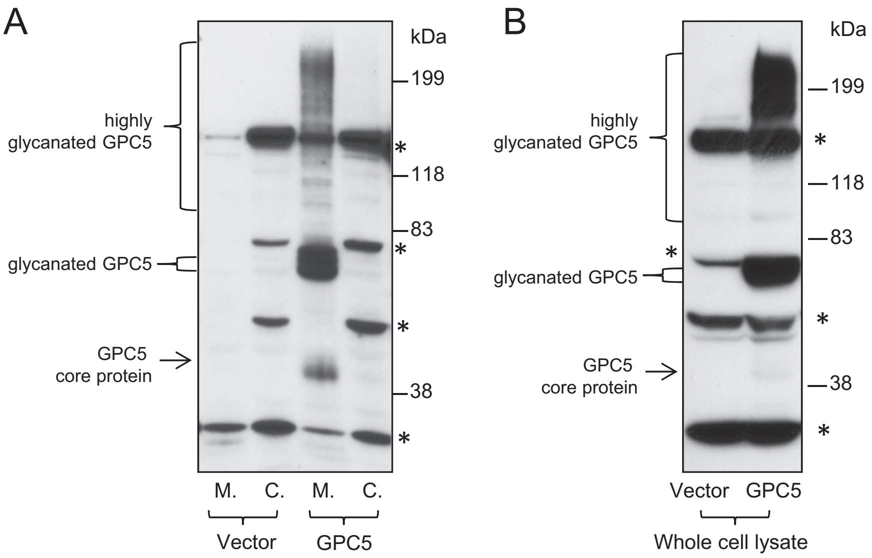

Fig. 1. GPC5 is localized exclusively to the membrane. (A) Western blotting of GPC5 for the membrane (M) and cytosolic (C) fraction of H1299 cells transfected with empty pcDNA3.1 vector of GPC5 cDNA. * indicates non-specific bands. (B) Western blotting of GPC5 for the whole cell lysate of H1299 cells transfected with empty pcDNA3.1 vector of GPC5 cDNA. * indicates non-specific bands.

2. Materials and methods

2.1. Cell culture and reagents

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (GIBCO) and incubated at 37 degree in a 5% CO_2 humidified incubator. Rabbit anti-E-cadherin, Tubulin, phospho-ERBB2, and phospho-ERBB3 antibodies were from Cell Signaling; rabbit anti-GPC5 (detects N-terminal AAs 46-61: RGLPDSPRAGPDLQVC) was from Biomatic; and goat anti-Actin was from Santa Cruz. All chemicals were from Sigma unless specifically indicated.

2.2. Transfection and antibiotic selection

GPC5 cDNA was from Origene and subcloned into the pcDNA3.1 plasmid (Invitrogen). GPC5 shRNAs were from Open Biosystems. For transient transfection, cells were plated at ~80% confluence one day before the transfection. Transfection was performed by using lipofectamine 2000 (Invitrogen). For stable transfection, antibiotic selection started 48 hours after transfection.

2.3. Preparation of cell lysate and western blotting

The membrane and cytosolic lysate was prepared by fractionation using Mem-Per plus membrane extract kit from Thermo Scientific. Non-fractionated whole cell lysate was prepared by lysing the cells directly in RIPA lysis buffer supplemented with PMSF, Na_3PO_4, and proteinase inhibitor cocktail (Santa Cruz). For Western blotting, 10-30 µg proteins were separated by SDS-PAGE and transferred onto PVDF membranes. After brief blocking in 5% skim milk, the membrane was incubated with primary antibodies, followed by HRP-conjugated secondary antibody incubation. Protein bands were visualized by Supersignal ECL substrates (Pierce).

2.4. Human phospho-receptor tyrosine kinase array

The human phospho-receptor tyrosine array kit was purchased from R&D (ARY001B) and performed as instructed by the manufacturer.

2.5. Statistics

Statistical significance was determined using two-sided Student's *t*-tests. P values less than 0.05 were considered statistically significant.

2.6. Xenograft experiment

Wild type 129/sv mice were purchased from Charles Rivers Inc. All protocols for mouse experiments were approved by the Mayo Clinic IACUC.

Briefly, ~80% confluent cultured cells (1 million cells per injection) were trypsinized, re-suspended in ice-cold PBS, and subcutaneously injected into the flanks of 8-10 weeks old wild-type 129/sv mice. Autopsies were performed at three weeks after injection.

3. Results

3.1. Detection of GPC5 in the cellular membrane fraction

To develop a biochemical approach for detecting cellular localization of GPC5, we transiently expressed a full length human GPC5 cDNA in a lung cancer cell line (H1299 cells), isolated both the membrane and the cytosolic cell fractions from the transfectants, and performed Western blotting for GPC5. The results showed that GPC5 was exclusively expressed in the membrane fraction of GPC5-transfected H1299 cells but was non-detectable in the cytosol (Fig. 1A). Endogenous GPC5 was also non-detectable in the control empty vector-transfected H1299 cells. Notably, besides the core protein (~40 kDa), several forms of glycanated GPC5 protein, including a major form of ~80 kDa and multiple smears ranging from 100 to 300 kDa (likely to be the highly glycanated forms) could be detected (Fig. 1A; asterisks indicate non-specific bands). Although Western blotting for non-fractionated whole cell lysates was able to detect both the core and glycanated GPC5, it failed to distinguish glycanated GPC5 from several non-specific bands (Fig. 1B, indicated by asterisks). Collectively, our results suggest that GPC5 is localized to the cellular membrane faction, which is consistent with its role as a GPI-anchored protein, and cell fractionation may be a useful approach for specifically

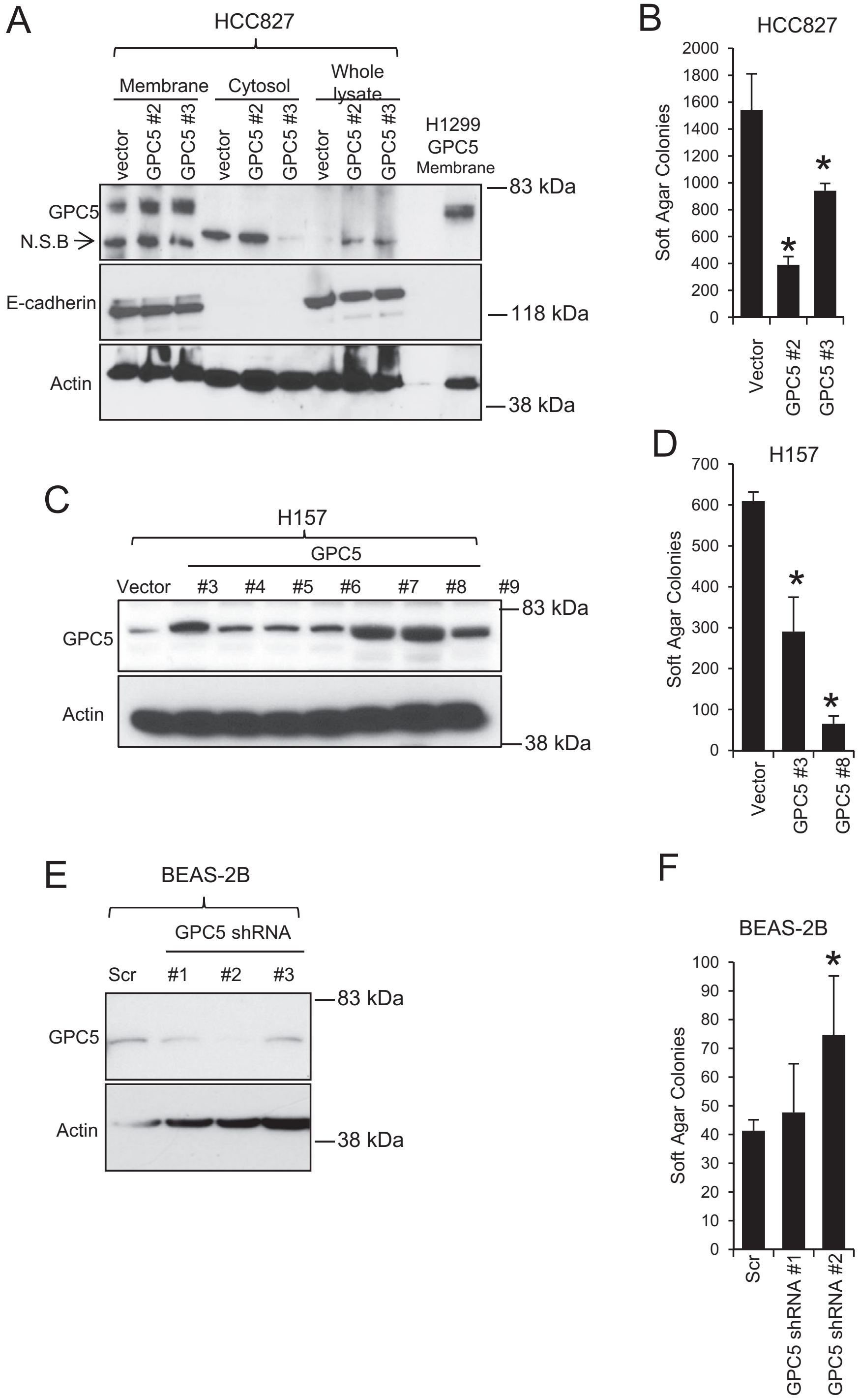

Fig. 2. GPC5 suppresses the tumorigenicity of lung cancer cells. (A) Western blotting of the whole cell lysate and the membrane and cytosolic factions of HCC827 cells stably expressing GPC5 or an empty pcDNA3.1 vector. The membrane protein E-cadherin was included as a control for successful membrane fractionation. Actin was included as a internal protein loading control. N.S.B.: non-specific band. (B) Soft agar colony formation assay for HCC827 cells stably expressing GPC5 or an empty pcDNA3.1 vector. (C) Western blotting for whole cell lysate of H157 cells stably expressing GPC5 or an empty pcDNA3.1 vector. (D) Soft agar colony formation assay for H157 cells stably expressing GPC5 or an empty pcDNA3.1 vector. (E) Western blotting for BEAS-2B cells stably expressing GPC5 shRNAs or control scrambled shRNA vector. (F) Soft agar colony formation assay for BEAS-2B cells stably expressing GPC5 shRNA or control scrambled shRNA vector. Note: in B, D, and F, * indicates *t*-test $p < 0.05$.

detecting membrane GPC5 and its glycanated forms.

3.2. *GPC5 suppressed lung cancer cell growth in Vitro and in Vivo*

To examine the role of GPC5 in lung cancer, we generated HCC827 and H157 lung cancer cells that stably overexpress GPC5 (Fig. 2A and C). We found that the overexpression of GPC5 significantly suppressed soft agar colony growth of both cell lines (Fig. 2B and D). On the contrary, stable knockdown of GPC5 in a human non-tumorigenic bronchial epithelial cell line (BEAS2B cells) promoted their soft agar colony formation ability (Fig. 2E), suggesting that loss of GPC5 may also facilitate the malignant transformation of the lung epithelia. Notably, unlike the transient GPC5 overexpressing H1299 transfectants (Fig. 1), all the above stable GPC5 transfectants only expressed the ~80 kDa glycanated GPC5, but not the core protein (~40 kDa) or the putative highly

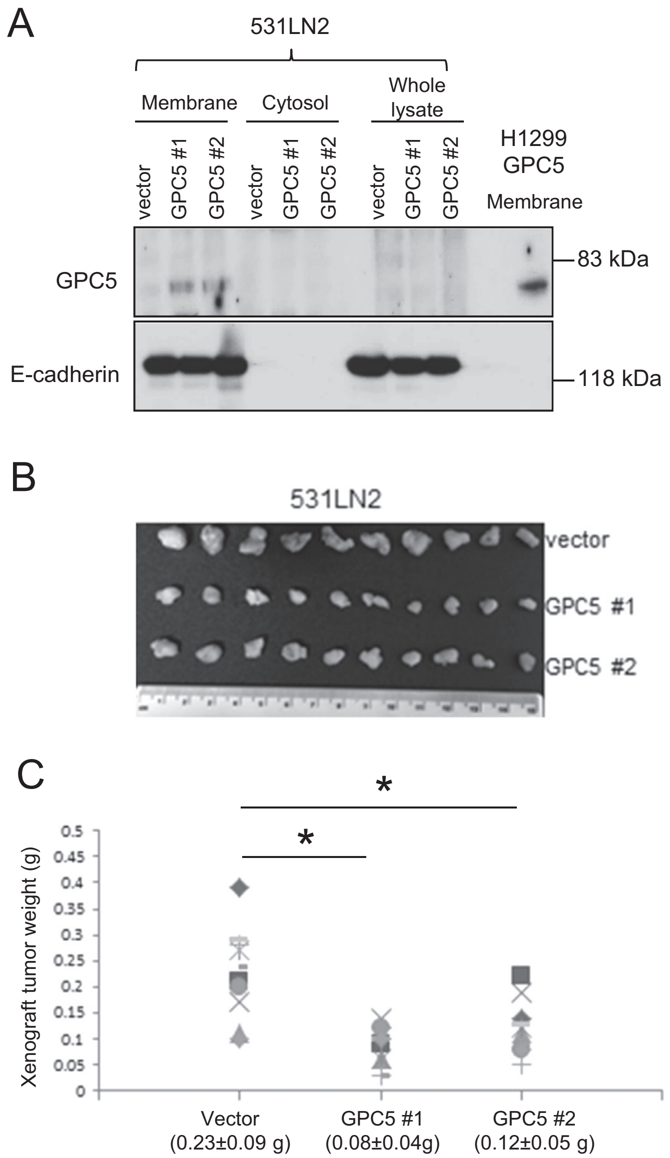

Fig. 3. GPC5 suppresses the xenograft tumor growth of 531LN2 lung adenocarcinoma cells. (A) Western blotting of the whole cell lysate and the membrane and cytosolic factions of 531LN2 cells stably expressing GPC5 or an empty pcDNA3.1 vector. The membrane protein E-cadherin was included as a control for successful membrane fractionation. (B and C) Photos (B) and quantification (C, by weight) of the xenograft tumors formed by 531LN2 cells stably expressing GPC5 or an empty pcDNA3.1 vector. Cells (1 million per injection) were subcutaneously into the flanks of 8-10 weeks old wild type 129/sv mice, and autopsy was performed at three weeks after the injection. Note: each of the symbols in Fig. 3C indicates an individual tumor. * indicates *t*-test $p < 0.05$.

glycanated form of GPC5 (~100–300 kDa), suggesting that the ~80 kDa glycanated GPC5 may be more stable than other forms of this proteoglycan. To determine the role of GPC5 *in vivo*, we utilized a syngeneic lung tumor model that we recently developed [17]. As shown in Fig. 3, overexpression of GPC5 in the 531LN2 lung adenocarcinoma cells (Fig. 3A and B) significantly suppressed their subcutaneous xenograft tumor growth (Fig. 3C). Collectively, these *in vitro* and *in vivo* results suggest that GPC5 acts as a tumor suppressor.

3.3. GPC5 suppressed receptor tyrosine kinases RYK and ERBBs

Previous studies have shown that the membrane-bound GPC5 may not function as a receptor for a specific ligand or growth factor. Instead, it may bind to and regulate the activity of other membrane receptors and their downstream signaling pathways [11,18]. In lung cancer, extensive studies have shown that receptor tyrosine kinases (RTKs), such as the EGFR/ERBB family of RTKs (EGFR and ERBB2-4), play oncogenic roles in lung tumorigenesis [19,20]. Thus, we performed a phospho-receptor tyrosine kinase array for the GPC5-expressing HCC827 cells. The results revealed that the tyrosine phosphorylation of several receptor tyrosine kinases, including RYK, ERBB2, and ERBB3 (Fig. 4A), were significantly suppressed by GPC5 expression. We also confirmed these findings by performing Western blotting for the GPC5-expressing 531LN2 cells (Fig. 4B). Interestingly, GPC5 dramatically depleted the expression of total ERBB2 and ERbb3 (Fig. 4B), indicating that it inhibits these ERBB family members by suppressing their expression. Together, these results suggest that GPC5 may exert its tumor suppressive function by repressing these oncogenic RTKs.

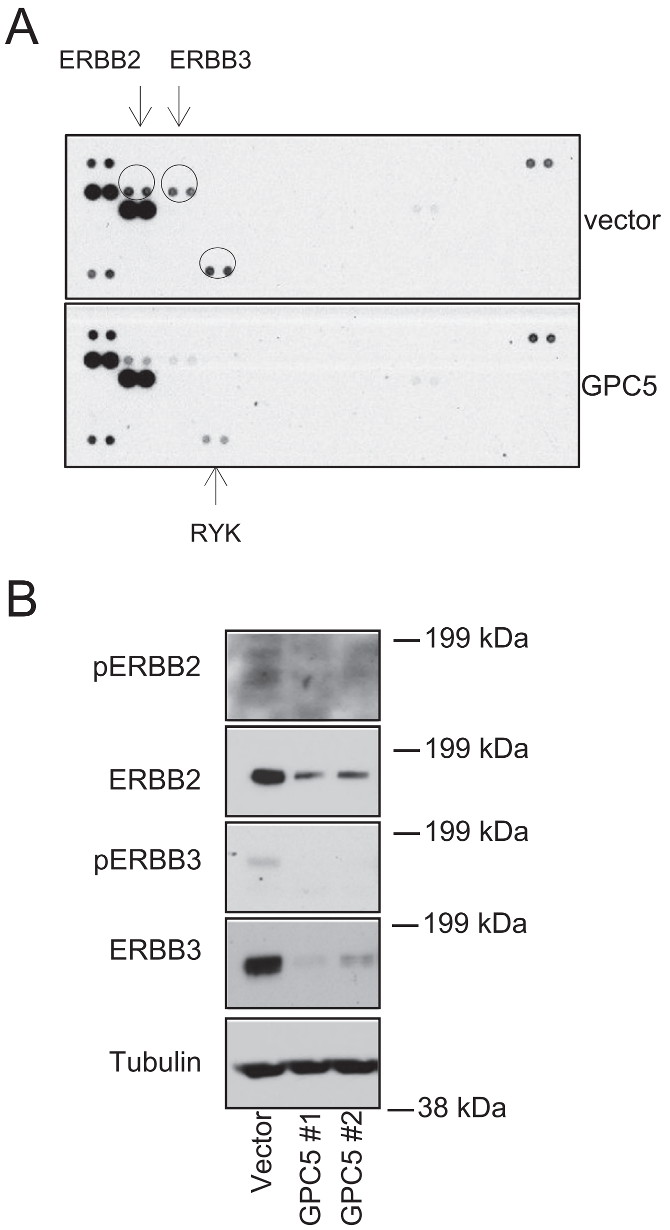

Fig. 4. GPC5 suppresses the tyrosine phosphorylation of oncogenic RTKs. (A) phospho-RTK array of HCC827 cells expressing GPC5 or an empty pcDNA3.1 vector revealed that GPC5 suppresses the phosphorylation of RYK, ERBB2, and ERBB3 (circled). (B) Western blotting for 531LN2 cells expressing GPC5 or an empty pcDNA3.1 vector.

4. Discussion

Although GPC5 was proposed to have a tumor suppressive function in lung cancer based on genetic and expression analyses [10,11], *in vivo* assessment of its role is lacking, and *in vitro* studies have led to completely opposite conclusions about its association with clinical outcome of NSCLC patients [15,16]. For instance, a recent report has shown that GPC5 overexpression promotes the migration of NSCLC cells, and high levels of GPC5 correlate with poor prognosis in NSCLC [16]. In contrast to this report, another study has shown that GPC5 inhibits NSCLC metastasis, and high levels of GPC5 correlate with better prognosis [15]. The biologic basis for such divergence is unclear. Here, we provide the first *in vivo* evidence that GPC5 acts as a tumor suppressor (Fig. 3). Interestingly, our results also revealed that multiple forms of GPC5 can be simultaneously detected (Fig. 1). It would be interesting to determine whether these different forms of GPC5 are present in patient tissues and have distinct prognostic values.

Our findings are also different from those using rhabdomyosarcoma cells or gastric cancer cells, where GPC5 may act as an oncogene to promote tumor cell growth by activating the Hedgehog signaling [21,22]. Notably, our results showed that GPC5 expression strongly suppressed several RTKs that are important for lung tumorigenesis, including the ERBB family members (ERBB2 and ERBB3), suggesting that GPC5 may regulate different signaling pathways in distinct types of cancer. Thus, targeting GPC5 may lead to distinct outcomes; while GPC5 antagonists may be useful to treat rhabdomyosarcoma or gastric cancer, reagents that activate GPC5 may be useful for treating NSCLC patients.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.03.010.

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