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REVIEW

Functions of the adaptor protein p66^{Shc} in solid tumors

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Abstract **p66^{Shc} is a 66 kDa Src homology 2 domain containing (Shc) adaptor protein homolog. Previous studies have demonstrated that p66^{Shc} is a proapoptotic protein involved in the cellular response to oxidative stress and in regulating mammalian lifespan. However, accumulating evidence also indicates its critical role in solid tumor progression. The expression of p66^{Shc} varies in different types of solid tumors, and it can paradoxically promote as well as suppress tumor progression, survival, and metastasis, depending on the cellular context. In this review, we discuss its functions in various solid tumors, the mechanisms by which it mediates the process of anoikis (detachment-induced cell death), and the epigenetic mechanisms that regulate its expression. These studies indicate the potential of p66^{Shc} as a novel prognostic marker and therapeutic target for the prevention of tumor progression and metastasis.**

Keywords adaptor protein, p66^{Shc}, anoikis, metastasis, autophagy

Introduction

p66^{Shc} is an isoform of ShcA, which belongs to the Shc adaptor protein family. ShcA is expressed as three isoforms, p46^{Shc}, p52^{Shc}, and p66^{Shc}, with molecular masses of 46, 52, and 66 kDa, respectively. In humans, all ShcA isoforms are encoded by the same gene, but their expression is regulated by different promoters (Pelicci et al., 1992; Luzi et al., 2000; Shen and Tsao, 2004). p66^{Shc}, p52^{Shc}, and p46^{Shc} share three functionally identical domains: an N-terminal phosphotyrosine binding domain (PTB), a collagen homology domain (CH1), and a C-terminal Src homology 2 domain (SH2) (Migliaccio et al., 1997; Luzi et al., 2000). p66^{Shc} is the longest ShcA isoform with an unique collagen homology domain (CH2) at the N terminus (Fig. 1). The CH2 domain is comprised of 110 amino acids, which is rich in glycine and proline residues. Two critical serine phosphorylation (S36 and S54) sites also reside in the CH2 domain (Migliaccio et al., 1997). S36 is a critical regulatory site for the apoptotic activity and oxidative stress response of p66^{Shc} (Foschi et al., 2001; Faisal et al., 2002; Pellegrini et al., 2005; Veeramani et al., 2005a; Galimov et al., 2014), and S54 is important for its stability (Khanday et al., 2006). A cytochrome *c* binding (CB) region is present between the CH2 and PTB domain in p66^{Shc} protein, which is primarily involved in the regulation of oxidative stress in the mitochondria (Giorgio et al., 2005).

p66^{Shc} has been extensively studied, primarily for its role in the cellular response to oxidative stress and in regulating mammalian lifespan. In mesenchymal progenitor and osteoblastic cell models, H_2O_2 activates a protein kinase C (PKC)-β/p66^{Shc}/NF-κB signaling cascade, wherein p66^{Shc} functions as an essential mediator of apoptosis induced by H_2O_2 in the osteoblastic cells (Almeida et al., 2010). Deletion of p66^{Shc} in mice (p66$^{Shc-/-}$) decreases the incidence of aging-associated diseases (e.g., atherosclerosis) and prolongs lifespan (Migliaccio et al., 1999; Napoli et al., 2003; Francia et al., 2004). p66^{Shc} is primarily expressed in epithelial cells; whereas, it is poorly expressed in peripheral blood lymphocytes, hematopoietic cell lines, and neurons. Its expression varies in different cancer cell lines (Pelicci et al., 1992; Xie and Hung, 1996; Migliaccio et al., 1997; Stevenson and Frackelton, 1998; Jackson et al., 2000; Veeramani et al., 2005a). Notably, the diverse expression of p66^{Shc} might be correlated with its differential functions in cells. Importantly, a growing number of studies have reported its involvement in cancer development, progression and metastasis (Alam et al., 2009). Herein, we review the different functions of p66^{Shc} in solid tumors, which may provide potential insight into cancer therapies targeting p66^{Shc}.

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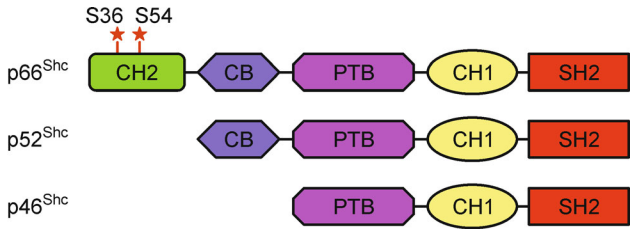

Figure 1 Schematic of ShcA. ShcA adaptor protein consists of three isoforms, p46^{Shc}, p52^{Shc}, and p66^{Shc}. They all contain three functionally identical domains, PTB, CH1 and SH2. p66^{Shc} contains cytochrome *c*-binding (CB) domain between the CH2 and PTB domains. At the N-terminal, p66^{Shc} also possesses a unique CH2 domain which contains vital serine phosphorylation sites (S36 and S54), with a proapoptotic effect of S36 phosphorylation and an effect of S54 phosphorylation for its stability, respectively.

Functions of p66^{Shc} in solid tumors

p66^{Shc} has been shown to promote cancer as well as suppress cancer. The expression of p66^{Shc} varies in different types of cancer. The expression of p66^{Shc} is elevated in many cancers, including prostate cancer (PCa) (Lee et al., 2004), esophageal cancer (Chen and Yang, 2001), thyroid cancer (Pelicci et al., 1995), ovarian cancer (OCa) (Muniyan et al., 2015), and colon cancer (Grossman et al., 2007). In contrast, the expression of p66^{Shc} is reduced in human lung cancers (Du et al., 2013; Zheng et al., 2013) and in malignant ovarian surface epithelial cells (Abdollahi et al., 2003). Differences in the expression of p66^{Shc} in various cancers may reflect differences in its biological functions in solid tumors.

p66^{Shc} promotes tumor progression

One of the strongest evidence that Shc is directly involved in cancer cell metastasis is provided by a study on transgenic mice strains expressing polyoma virus middle T antigen with a mutated Shc binding site (Webster et al., 1998). Female transgenic mice developed focal mammary tumors, but surprisingly, in a number of metastatic tumors, the mutated Shc binding site had reverted to the wild-type sequence. This result suggests that the process of metastasis provides a strong selection pressure for a functional Shc binding site *in vivo*. This study also implicated p66^{Shc} in specific steps of metastasis. The expression and activation of p66^{Shc} was elevated in a highly metastatic variant (F-11) of the human breast cancer cell line, MDA-MB-231, compared to the parental cell line (Jackson et al., 2000). Moreover, in breast cancer tissues associated with lymph node metastasis, p66^{Shc} expression was elevated, which correlated with a greater number of positive nodes (Jackson et al., 2000). These results suggest that cell motility and invasion are influenced by p66^{Shc} rather than by the MAPK pathway (Jackson et al., 2000). Conversely, reduced ShcA levels or the expression of a dominant-negative ShcA mutant limited TGF-β-induced motility and the invasion of Neu/ErbB2-expressing breast cancer cells (Northey et al., 2008). Together, these results suggest a critical role of p66^{Shc} in the migration and invasion of cancer cells.

p66^{Shc} may also play a critical role in steroid-stimulated cancer cell proliferation. Steroid hormone-related cancers include cancers of the prostate, testes, breast, ovary, uterine endometrium, and thyroid (Henderson and Feigelson, 2000). In PCa, overexpression of p66^{Shc} increases basal cell proliferation. Conversely, p66^{Shc} knockdown with small interfering RNAs reduced dihydrotestosterone (DHT)-induced cell proliferation. A novel role for the p66^{Shc}-ROS (reactive oxygen species) pathway in androgen-induced cell proliferation has also been reported (Veeramani et al., 2008). Androgens induce the production of ROS in PCa cells through the increased expression of p66^{Shc}, resulting in the inactivation of tyrosine phosphatase activity required for the activation of an interacting tyrosine kinase and increased cell proliferation and tumorigenicity (Veeramani et al., 2012). Similarly, the regulation of OCa cell proliferation by p66^{Shc} has also been investigated. Among the cells lines tested, the slowest growing, OVCAR-3, expressed the lowest endogenous levels of p66^{Shc}, and overexpression of p66^{Shc} resulted in increased growth and proliferation (Muniyan et al., 2015). Treatment of OCa cells with steroids resulted in an upregulation of p66^{Shc} levels and increased cell proliferation; conversely, treatment with steroid receptor antagonists resulted in a downregulation of p66^{Shc} levels (Alam et al., 2009). In CaOV-3 cells treated with estrogen (E2), p66^{Shc} protein levels were elevated, which correlated with increased ROS production, ErbB2 and extracellular signal-regulated kinase (ERK)/MAPK activation, and cell proliferation (Muniyan et al., 2015). p66^{Shc} forms a trimeric complex with alpha-1-syntrophin and Grb2, which can trigger cell proliferation and migration in MCF-7 and HBL-100 breast cancer cell lines; knockdown of p66^{Shc} using siRNAs decreased cell proliferation. Together, these results (Bhat et al., 2014) clearly establish a causal relationship between p66^{Shc} protein and cell proliferation.

p66^{Shc} acts as a tumor suppressor

Despite considerable evidence demonstrating that p66^{Shc} promotes tumor progression, p66^{Shc} can also function as an important tumor suppressor by inhibiting cancer cell survival and metastasis. Recent studies have shown that p66^{Shc} regulates cancer cell apoptosis or anoikis (detachment-induced cell death). For example, phenethyl isothiocyanate (PEITC) treatment of PC-3 and LNCaP PCa cells resulted in the induction of p66^{Shc} expression, phosphorylation of S36, and selective inhibition of the growth of PCa cells by inducing apoptosis. Furthermore, PEITC treatment increased the binding of p66^{Shc} with peptidyl-prolyl isomerase (Pin1), and induced the translocation of p66^{Shc} to the mitochondria (Xiao and Singh, 2010). In addition, p66^{Shc} is a stronger

mediator of pro-death signaling in PC-3 cancer cells than in the PNT1A cells (noncancerous human prostate epithelial cells) after diallyl trisulfide (DATS) treatment (Borkowska et al., 2013). Moreover, $p66^{Shc}$ deficiency increases apoptosis resistance by nutrient deprivation in human lung adenocarcinoma A549 cells (Zheng et al., 2013). $p66^{Shc}$ also functions as a focal adhesion protein, and mediates anoikis in epithelial cells through RhoA-dependent anchorage sensing (Ma et al., 2010). $p66^{Shc}$ is downregulated in mouse Lewis lung carcinoma (LLC) and two cell lines of human small cell lung cancer (SCLC; H69 and H209). These cells exhibit aggressive metastatic behavior, presumably by bypassing anoikis through the constitutive activation of Ras. Interestingly, re-expression of $p66^{Shc}$ in these cells restores anoikis (Ma et al., 2010). These results suggest that $p66^{Shc}$ may act as an important tumor suppressor. In clinical human lung cancer samples and cancer cell lines, the promoter of $p66^{Shc}$ is hypermethylated at specific CpG sites in the early post-transcriptional region of $p66^{Shc}$. Hypermethylation silences its expression and may contribute to the invasion and metastasis cascade (Du et al., 2013). Thus, the epigenetic repression of $p66^{Shc}$ in cancer cells might be the key to the upregulation of Nrf2, and enhanced cell survival and tumor progression. The epithelial-to-mesenchymal transition (EMT) program is crucial for epithelial cancer cell progression. We have found that $p66^{Shc}$, which regulates ZEB1 within a negative feedback loop, suppresses fibrotic EMT responses (Li et al., 2015). ZEB1, a well characterized EMT transcription factor and an activator of EMT, promotes tumorigenicity and metastasis. The expression of $p66^{Shc}$ represses ZEB1. The $p66^{Shc}$ promoter is also inhibited by ZEB1, which can induce fibrotic EMT responses and increase cell invasion and migration in lung cancer cells. Aiolos, a hematopoietic transcription factor, can promote cancer cell survival in an unanchored state by altering the chromatin structure surrounding the *SHC1* gene, leading to an isoform-specific silencing of $p66^{Shc}$ (Li et al., 2014). Our studies reveal in depth mechanisms by which $p66^{Shc}$ prevents metastatic behavior.

Functional mechanism of $p66^{Shc}$

$p66^{Shc}$ regulates anchorage dependence and mediates anoikis

Integrin-dependent attachment of parenchymal cells to solid structures is required for their survival. As a consequence of anchorage dependence, most tissue cells initiate apoptotic death within hours of being suspended in a fluid environment, a process termed anoikis (Frisch and Francis, 1994). Physiological anoikis maintains homeostasis in developing and adult tissues (Mailleux et al., 2007); whereas, pathological anoikis resistance is implicated in malignant cell metastasis (Ma et al., 2010).

Presently, anoikis is thought to be regulated by the withdrawal of integrin-related outside-in survival signals. This is supported by the observation that the enforced activation of various signaling components downstream of integrin signaling pathways are sufficient to confer anoikis resistance (Frisch and Francis, 1994; Frisch et al., 1996; Gilmore, 2005; Martin et al., 2006). The binding of extracellular matrix components alone is not sufficient to prevent anoikis, since in the absence of structural matrix rigidity, RGD (Arg-Gly-Asp)-mediated integrin binding does not prevent anoikis (Re et al., 1994; Chen et al., 1997). Thus, until recently, the molecular basis for attachment sensing during anoikis has been poorly understood. However, we recently discovered that $p66^{Shc}$ permits activation of RhoA, resulting in tension-dependent death of suspended cells (Ma et al., 2007). Anoikis sensitivity correlates with the upregulation of $p66^{Shc}$, induced by either external or endogenous mechanisms, in endothelial, epithelial, and mesenchymal cells. Lack of $p66^{Shc}$ bypasses anoikis, whereas re-expression of $p66^{Shc}$ restores anoikis. $p66^{Shc}$ localizes to focal adhesions in attached cells and results in the focal activation of RhoA GTPase at regions of integrin anchorage. Because RhoA increases tension at integrin attachment sites (Nobes and Hall, 1995), in detached cells, the tension would be applied among unanchored sites, allowing a mechanical readout for detachment. Upon ECM detachment, this $p66^{Shc}$-dependent tension test detects a load failure, leading to RhoA-dependent anoikis (Ma et al., 2007). Nevertheless, the mechanism by which unopposed tension causes cell death is unclear. In metastatic cancer cells, $p66^{Shc}$ mediates attachment sensing and anoikis. Knockdown of $p66^{Shc}$ in lung tumor cells leads to unrestrained Ras activation, resulting in the downstream suppression of RhoA and prevention of anoikis (Ma et al., 2010). The small-cell lung cancer and LLC cell lines, which lack $p66^{Shc}$, exhibit constitutive K-Ras activation, which can be suppressed by the ectopic expression of $p66^{Shc}$. Our data further suggest that $p66^{Shc}$ restrains the hyperactivation of Rac1 and deactivation of RhoA downstream of Ras. Downstream Ras-dependent survival signals (e.g., Akt and ERK) may also contribute to anoikis resistance (Ma et al., 2010).

Aiolos suppresses $p66^{Shc}$

Aiolos is frequently expressed in lung cancers and is correlated with markedly reduced patient survival. In lung cancer tissues and isolated single cells, the expression of $p66^{Shc}$ is inversely correlated with that of Aiolos, which confers anoikis resistance and promotes lung cancer metastasis (Li et al., 2014). Aiolos, a member of the Ikaros zinc finger family, is a lymphocyte lineage-restricted transcription factor. Its expression is generally restricted to lymphoid cells (Morgan et al., 1997). Aiolos is involved in hematopoietic cell development. Aiolos is detected at low levels in pro-B and double negative ($CD4^-$ $CD8^-$) thymocyte precursors but

is upregulated in cells as they progress to pre-B and double positive ($CD4^+$ $CD8^+$) stages of differentiation. Aiolos expression peaks in mature peripheral B cells (Wang et al., 1998; Thompson et al., 2007). Notably, bioinformatic analysis of the transcriptome of some human breast cancers shows that Aiolos expression is upregulated (Kilpinen et al., 2008). However, the function of Aiolos in carcinomas has not been well characterized. Our microarray analysis shows that multiple genes correlated with cellular adhesion are downregulated in A549 cells re-expressing Aiolos (Li et al., 2014). Transient expression of Aiolos also selectively decreased p66^{Shc} protein levels in A549, HepG2, and MDA-MB-231 cells, confirming Aiolos inhibits p66^{Shc} expression. Furthermore, p66^{Shc} transcription requires a long-range physical interaction between the primary enhancer, E2, and the p66^{Shc} promoter in lung cancer cells. Aiolos associates with E2 and nearby surrounding regions, resulting in the alteration of higher order chromatin structures, disruption of enhancer-promoter interactions, and silencing of p66^{Shc} transcription. Thus, Aiolos contributes to anoikis resistance *in vitro* and *in vivo*.

p66^{Shc} and ROS in solid tumors

p66^{Shc} modulates ROS production (Nemoto and Finkel, 2002; Giorgio et al., 2005; Khanday et al., 2006), which is mainly produced by mitochondrial respiration (Veeramani et al., 2012; Galimov et al., 2014). ROS induces oxidative damage, which may result in tissue dysfunction (Trinei et al., 2013). In the mitochondrial intermembrane space, p66^{Shc} binds to cytochrome *c*, acting as an oxidoreductase and generating ROS (Nemoto and Finkel, 2002; Giorgio et al., 2005). In addition, p66^{Shc} also mediates the production of ROS from the nucleus and plasma membrane (Nemoto and Finkel, 2002; Khanday et al., 2006). In turn, elevated ROS level induces the phosphorylation of p66^{Shc} protein at S36, further promoting ROS generation (Nemoto and Finkel, 2002; Orsini et al., 2004). ROS generated by p66^{Shc} has multiple functions in carcinogenesis. First, ROS may contribute to tumor cell proliferation. Stimulation of PCa cells with androgens induced the production of ROS through the elevation of p66^{Shc} protein levels, resulting in increased cell proliferation and enhanced tumorigenicity (Veeramani et al., 2012). ROS produced by p66^{Shc} inactivates cellular prostatic acid phosphatase (cPAcP), which is a biomarker of PCa (Sakai et al., 1992; Varma et al., 2004) and which inhibits PCa growth as a protein tyrosine phosphatase (PTPase) (Lin and Meng, 1996; Lin et al., 2001; Veeramani et al., 2005a; Veeramani et al., 2005b). Inhibition of cPAcP then activates ErbB2 and ERK/MAPK, which promote cell growth, survival and tumorigenicity (Veeramani et al., 2005a). Increased p66^{Shc} levels and ROS production contribute to cell growth in 5α-dihydrotestosterone (DHT)-treated PCa cells as well (Veeramani et al., 2008). Second, the pro-apoptotic effects of ROS are well known. ROS accumulation is secondary to the progress of the apoptotic process. Accumulated ROS can activate the apoptotic pathway, which includes the collapse of the mitochondrial transmembrane potential (Malhotra et al., 2009) and release of proapoptotic factors, such as cytochrome *c* (Giorgio et al., 2005). PEITC selectively inhibits the growth of human PCa cells by inducing ROS production and apoptosis, for which p66^{Shc} is indispensable (Xiao and Singh, 2010). PEITC treatment enhances PKC_β-mediated S36 phosphorylation of p66^{Shc}, which increases its association with Pin1 and promotes its translocation to the mitochondria. p66^{Shc} protein, localized to the mitochondria, is released from HSP70 and stimulates ROS production, leading to caspase-3 activation and apoptotic cell death (Xiao and Singh, 2010). Therefore, the production of ROS by p66^{Shc} plays a controversial but critical role in cancer cell survival and death.

p66^{Shc} induces autophagy

Autophagy is an evolutionarily conserved lysosome-dependent process, wherein cytosolic components are enzymatically degraded and recycled in response to a wide variety of stressful conditions including ionic dysfunction, protein aggregation, proteasome failure, and energetic oxygen and nutrient deprivation (Levine, 2007; Deffieu et al., 2009). The role of autophagy in cancer is complex and controversial. Autophagy contributes to the survival and growth of cancer cells under stressful conditions. For instance, serum starvation induces autophagy and inhibits apoptosis in SHSY5Y cells through the upregulation of NF-κB and Bcl-2 and downregulation of Bax and caspase-3 (Mohan et al., 2011). Under environmental metabolic stress, lysosomal associated transmembrane protein, LAPTM4B, is elevated, which increases autophagic flux and promotes breast tumor growth *in vivo* (Li et al., 2011). However, autophagy can also limit the survival and growth of cancer cells. In several tumor types, autophagy deficiency may positively correlate with the tumorigenesis when Atgs are mutated (Wirawan et al., 2012). The cytoprotective enzyme, Heme oxygenase-1 (HO-1), can reduce autophagy and promote the survival of tumor cells against chemotherapy (Banerjee et al., 2012).

p66^{Shc} may be involved in the process of autophagy induced by nutrient deprivation (Zheng et al., 2013). Non-small lung cancer A549 cells express high basal levels of phosphorylated ERK1/2 (Thr202/Tyr204) and phosphorylated Akt1(Ser473), which decrease with nutrient deprivation. ERK1/2 are conserved serine/threonine kinases that regulate many cellular programs, including autophagy (Pattingre et al., 2003). High constitutive ERK activity is implicated in suppressing autophagy in some cancers. Aberrant sustained activation of ERK by the carcinogen, Lindane, disrupts the maturation of autophagosomes into functional autolysosomes (Yang et al., 2013). The oncogene, Akt, negatively regulates autophagy by positively regulating the activity of the mTORC1 complex (Levine, 2006; Guertin and Sabatini,

2007). p66^{Shc} deficiency mitigates but does not completely inhibit low-nutrient-induced autophagy in lung cancer A549 cells (Zheng et al., 2013). This requires prolonged activation of ERK1/2 (Thr202/Tyr204) but not of phosphorylated Akt1 (Ser473) (Zheng et al., 2013). p66^{Shc} is necessary to promote autophagosome formation rather than reducing the autophagosome-lysosome fusion (Zheng et al., 2013). Oncogenic Ras negatively regulates autophagy (Levine, 2006). Ras signaling can also exert proneoplastic effects through the down-regulation of Beclin1, thus suppressing autophagy (Yoo et al., 2010). The loss of p66^{Shc} in lung tumor cells leads to unrestrained Ras activation (Ma et al., 2010; Zheng et al., 2013). Therefore, K-Ras may be involved in p66^{Shc}-dependent autophagy in nutrient-poor conditions. In nutrient-deprived A549 cells, knockdown of p66^{Shc} decreases LC3B-I to-II conversion, the number of autophagic vacuoles, and p62/sequestosome 1 protein degradation (Zheng et al., 2013). In addition, p66^{Shc} depletion mitigates the process of autophagy induced by ECM detachment; however, the exact mechanism has not been characterized. In addition to its functions in tumor cells, p66^{Shc} can function as a discrete but essential mediator of metabolic tone and autophagosome formation in neurons. p66^{Shc}-mediated autophagy acts as an adaptive mechanism to promote the removal of injured neuronal organelles following low level oxygen and glucose deprivation (Brown et al., 2010).

Future directions and perspectives

The expression of p66^{Shc} varies in different types of solid tumors. In steroid-induced epithelial cancer cells, p66^{Shc} protein levels are elevated; whereas, p66^{Shc} protein levels are decreased in human lung cancers. This difference may result from the different functions of p66^{Shc} in tumor cells. Although the mechanisms that underlie p66^{Shc} involvement in tumor progression, anoikis and autophagy in tumor cell have been investigated (Fig. 2), the exact mechanisms and upstream regulators and downstream effectors of p66^{Shc} functional pathways warrant further investigation.

Nevertheless, these paradoxical properties of p66^{Shc} make it a novel prognostic and therapeutic target for preventing tumor progression and metastasis. For example, cPAcP, which is a biomarker of PCa and which inhibits tumor cell growth, is inactivated by ROS produced by p66^{Shc}. Thus, reducing p66^{Shc} protein by upregulating its ubiquitination is a potential therapeutic approach to inhibit PCa cell proliferation. Conversely, Aiolos, a transcription factor, alters the chromatin structure surrounding the *SHC1* gene and silences

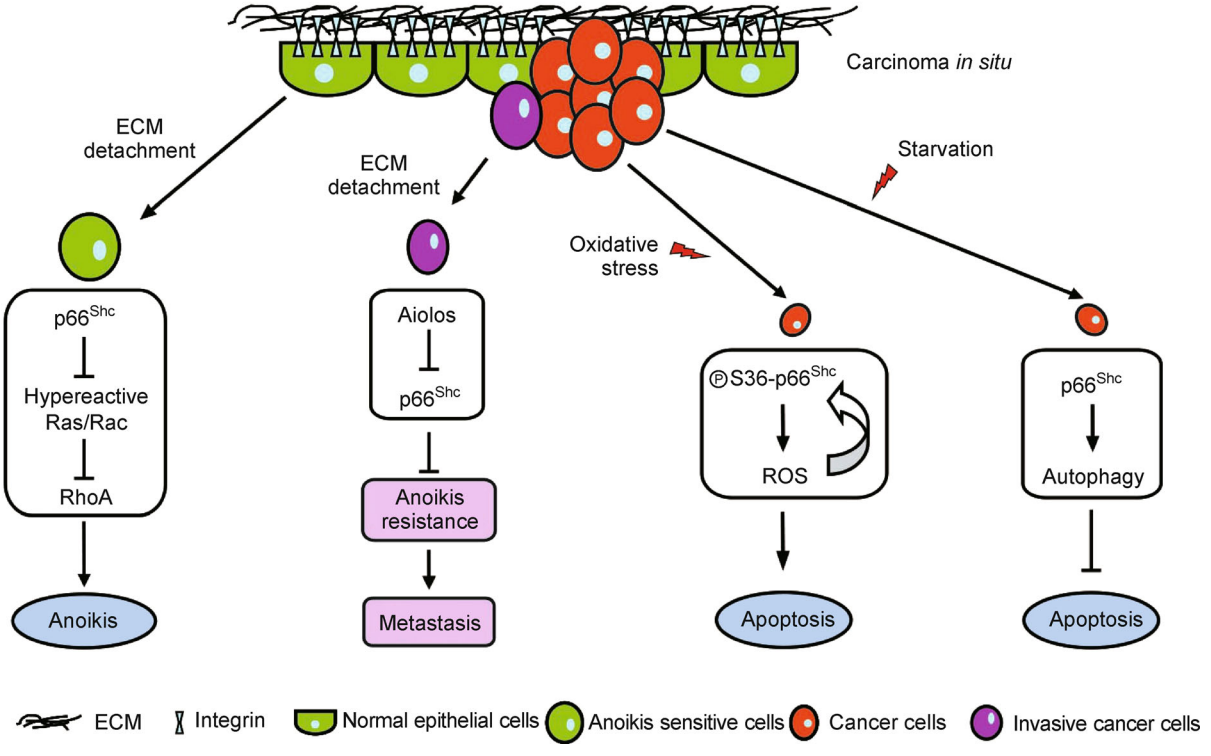

Figure 2 Functional mechanisms of adaptor protein p66^{Shc} in solid tumors. In normal epithelial cells of solid tumor tissue, p66^{Shc} regulates anchorage dependence and mediates anoikis by inhibiting Ras hyperactivation, activating RhoA when losing attachment to ECM. Conversely, in some cancer cells within the ability of invasion, aberrantly expressed Aiolos suppresses p66^{Shc} expression, conferring anoikis resistance and promoting tumor metastasis in an unanchored state. Under oxidative stress, p66^{Shc} may function a proapoptotic role and promotes apoptosis through the forced loop between S36 phosphorylation and ROS production. However, under nutrient-deprivation stress, dysfunctional p66^{Shc} protein triggers autophagic survival, leading to apoptotic resistance of cancer cells.

the expression of p66^{Shc}, which functions as a metastasis suppressor. Thus, in this case, reactivation of p66^{Shc} by regulating Aiolos-p66^{Shc} interactions may be a candidate therapeutic approach for metastatic lung cancer.

Compliance with ethics guidelines

Yanan Sun, Jie Yang and Zhenyi Ma declare that they have no conflicts of interest.

This manuscript is a review article and therefore does not require the approval of the relevant institute review board or ethics committee.

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