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Review

Ki-67 protein as a tumour proliferation marker

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ABSTRACT

Newer treatment strategy based on proliferative nuclear marker Ki-67 targeted therapy holds promise for prioritized/personalized treatment options with regard to improved survival and outcome in patients with renal cancer. Over the past decade, the importance of Ki-67 in prognosis of breast cancer has been widely studied, however very few studies and literatures are available in the context of renal cancer which has an increasing incidence internationally. The focus of this present review is to fill the gaps pertaining to its prognosis and management with newly understood mechanisms of targeted interventions. Recent breakthrough discoveries have highlighted the correlation of Ki-67 expression to stage and metastatic potential in renal tumours. A better understanding of molecular structure and different protein domains along with its regulation will provide evidence for precise target thereby controlling the proliferation rate correlated with decrease in the Ki-67 protein levels. Therapies targeting Ki-67 is still in the preclinical stage, besides its diagnostic and/or prognostic significance, a better understanding of targeted strategical studies is required for extrapolation to the clinical use. Current understanding of the associated molecular pathways and the precise role of Ki-67 could streamline the basis for predicting renal cancer outcome.

1. Introduction

Kidney cancer or renal cancer accounts for > 65,000 new cases diagnosed and > 14,000 deaths in the United States alone in both the sexes [1]. Renal cancer which arise in the renal parenchyma can be categorised as adenocarcinomas also referred to as renal cell carcinomas (RCC) accounting for upto 90% of adult kidney carcinomas, while those which arise from the collecting system are mainly referred to as transitional cell carcinomas (TCC). Renal transitional cell carcinoma (RTCC) are those which arise in the renal pelvis and constitute only 10% of histologically confirmed kidney carcinoma. The most common form of renal cancer in children is the nephroblastoma (Wilms tumour). Among renal cell carcinoma the predominant is the clear cell subtype followed by renal cell carcinoma not specified, papillary and chromophobe subtypes [2]. Diagnosis of renal cancer by means of molecular marker such as Ki-67 is a potential promising tool to evaluate the state of the disease. Earlier Fuhrman nuclear grading wherein nuclear size, nuclear pleomorphism and nucleolar prominence are taken into account for grading tumours. This techniques suffers intra and inter observer variation along with no clear guidelines laid out for grading renal tumours. In those cases, Ki-67 behave as a surrogate for nuclear grade and also objective interpretation could be carried out.

The Ki-67 protein was originally defined by 359 kDa monoclonal antibody Ki-67, obtained by immunising mice with the nuclei of the Hodgkin's lymphoma cell line L248 first identified by Gerdes et al. [3] in the year 1991. The name Ki-67 was first defined by its city of origin (Kiel) and the number of original clone (i.e. 67th clone) in the 96 well plate. The antigen was not initially characterised and hence the name Ki-67. Ki-67, a nuclear DNA binding protein which is expressed in all the vertebrates is a widely used marker of proliferation used for grading tumours [4]. Numerous reports from the cell cycle analysis in the nuclei has showed the presence of Ki-67 in the G_1, S and G_2 phases of the cell cycle and not in the quiescent or resting cells G_0 phase suggestive of its role as cell proliferation marker in many cancers [5]. Ki-67 labelling index as determined by immunohistochemistry (IHC) analysis on paraffin embedded section and percent positive frequency is indicative of patient outcomes. High Ki-67 index generally shows poor prognosis in clinical conditions [6].

Recently, Ki-67 has drawn increasing attention as an attractive prognostic, prediction and potential therapeutic target in malignant

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neoplasms associated with lung, bladder, breast, cervical, urothelial carcinomas, upper urinary tract, lymphoma and cervical cancer [7–12]. However the role of Ki-67 in renal cancers still remain ambiguous and were not thoroughly investigated. Therefore the current reviews intends to throw light on the importance of Ki-67 as a potential proliferation marker in emerging incidences of renal cancer and to investigate its prognostic and therapeutic potential.

2. Biology of Ki-67

2.1. Structure

Ki-67 is a non- histone nuclear and nucleolar protein encoded by *MKI-67* gene mapping to chromosome 10q26.2 in humans and has total exon count of 16. The Ki-67 gene is comprised of two different protein isoforms which are generated by alternative splicing of an mRNA precursor with or without exon 7 with molecular weight of 320 kDa and 359 kDa [13–15]. Molecular cloning of the pKi-67 showed the presence of 16 concatenated direct repeats (Ki repeats) of 122 amino acid length which is encoded by a single 6842 bp exon. Each repeat consist of highly conserved amino acid residues such as cysteine, glycine and glutamic acid which may possess functional property. Each repeat has highly conserved motif encoded by 66 bp element and also a number of PEST sites, region rich in proline (P), glutamic acid (E), serine (S) and threonine (T) which are susceptible to protease activity. The Ki-67 motif is the epitope recognised by the Ki-67 antibody such as MIB-1, routinely used to process human tissue. Due to this property Ki-67 has shorter biological half-life of < 1 h as confirmed by inhibitor studies [13,16]. Several protein phosphorylation sites are also present in Ki-67 like protein kinase C sites, casein kinase II sites, tyrosine kinase and consensus sites for cyclin dependent kinase 1 (CDK1) [13]. The N-terminus region consist of the forkhead-associated domain (FHA) which serves as a binding site for many transcription factors along with Protein Phosphatase 1 (PP1)-binding motif (PPD-1) and conserved domain (CD) of 31 amino acid with unknown function (Fig. 1). PPD-1 has important role in the phospho-regulation of nucleophosmin/B23 by Casein Kinase II (CKII) [17]. The C-terminus region consist of putative ATP/GTP-binding domain and LR domain comprising of 2896 amino acids which has a primary role in chromosome targeting of Ki-67 by its DNA binding ability [18,19]. Studies have reported that LR domain has the ability to change the higher order chomatin structure. C-terminus region also consist of the Kon21 domain which is involved in the maintenance/establishment of heterochromatin domains [20].

2.2. Functions of Ki-67

Ki-67 is mainly localized in the perinucleolar region in the G1 phase. During interphase Ki-67 localize to the dense fibrillary component of the nucleolus [21–23]. During mitosis as the nucleoli breakdown diffuse staining patterns were revealed which indicates its association with the chromatin. As chromosome condensation take place Ki-67 are found at the perichromosomal layer along with other nucleolar proteins like nucleophosmin (B23), fibrillarin, nucleolin, and perichromonuclein. The perichromosomal layer function as a protective sheath or pellicle around the chromosomes [24] which serve as a platform during nucleolar assembly [25]. A notable feature of Ki-67 is to localize granular nucleolar components to mitotic chromosomes and therefore play an important role in nucleolar segregation between daughter cells [17]. Researchers claim that Ki-67 functions as a surfactant which enable chromosome motility and its interaction with mitotic spindle thereby preventing the chromosome from collapsing into chromatin mass after nuclear envelope disassembly [26]. Studies have shown that Ki-67 has roles in rRNA transcription. These features during ribosome biogenesis may possibly explain the link between cell proliferation and Ki-67 expression [27]. Recent reports have suggested that Ki-67 does not directly correlate with cell proliferation however it is involved in the heterochromatin compaction and organisation possibly by histone methylation complexes required for heterochromatin maintenance [15].

2.3. Regulation of Ki-67 expression

In cell cycle the proliferation signature was first characterised by comparing the gene expression profile of human tumour with that of normal cell line which had undergone cell cycle arrest and proliferation [28]. Studies carried out with breast tumour showed increased expression patterns of E2F (cell cycle transcriptional regulator), MCM2-MCM6 (replication-initiation complex proteins minichromosome maintenance 2–6) which is indicative of tumour proliferation state measured by Ki-67 labelling index. Flow cytometry analysis revealed that levels of Ki-67 increase from late G1 phase to S phase and peaking at mitosis followed by sharp decline by the end of mitosis [29,30]. Numerous studies have shown that Ki-67 is regulated by phosphorylation. Briefly during interphase the non-phosphorylated form of Ki-67 form complex with DNA which is suggestive of its role in organisation of nucleolar chromatin in proliferating cells. However in the hyperphosphorylated form it does not bind to DNA during mitosis as shown by experiments indicating increased mobility [31]. Stabilisation and maintenance of mitotic bipolar spindle is achieved by recruiting kinesin-like motor protein, Hklp2 to mitotic chromosomes [32]. Another interaction specific to mitosis include Ki-67 and RNA-binding protein, Nucleolar protein interacting with the FHA domain of Ki-67 (NIFK) but the function is not clear [33]. Studies have confirmed that cell cycle dependent phosphorylation is tightly coupled to the breakdown of nucleus at the beginning of mitosis and gradually reverse as the sister chromatid separation completes. Human analog of cell division cycle 2 (cdc2), CDK-1 is considered as the key regulatory kinase which control the entry into mitosis. Posttranslational events such as phosphorylation

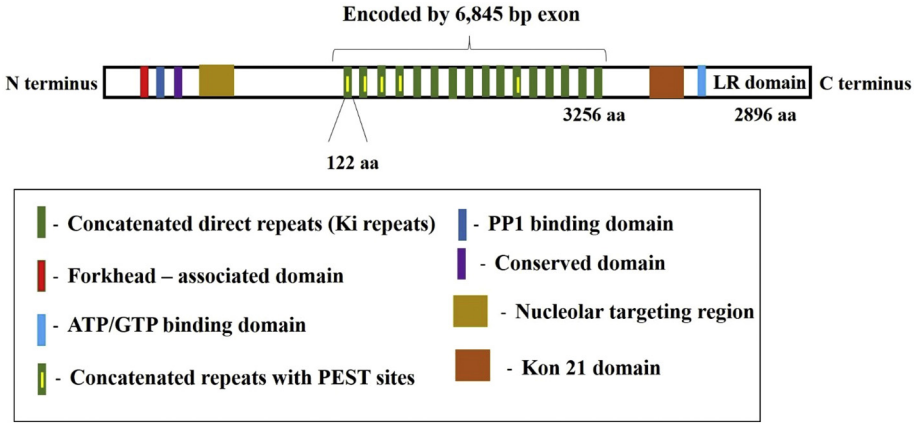

Fig. 1. Domain structure of human Ki-67. Ki-67 consist of 16 concatenated direct repeats of 122 amino acids with numerous PEST sites susceptible to proteolytic degradation each responsible for the antigen detection by the MIB-1 antibody. The N terminus region mainly consist of the FHA domain, PP-1 binding domain and conserved domain each serve different function. Several nucleolar targeting regions are also found. C terminus predominantly consist of the Kon 21 domain required for maintenance of heterochromatin, ATP/GTP binding domain and leucine arginine (LR) domain of 2896 amino acid.

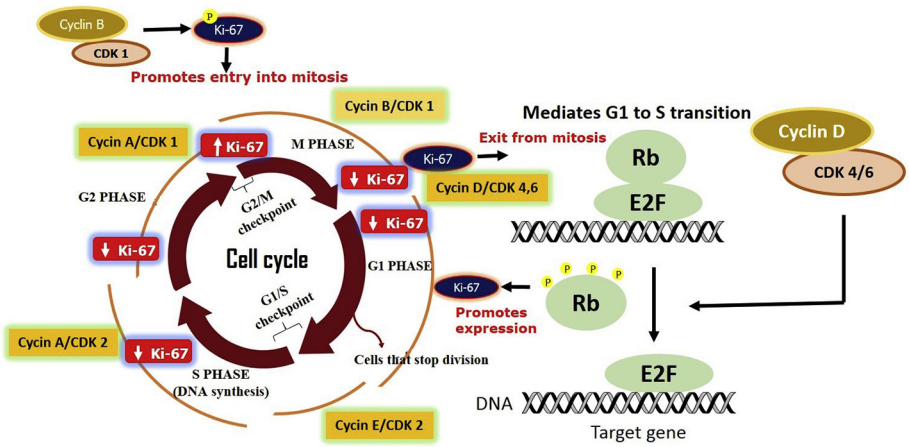

Fig. 2. Regulation of cell cycle by Ki-67. Ki-67 is present in all the phases of cell cycle except during resting or the G0 phase. Varying levels of Ki-67 range from G1 phase to M phase with highest found in the start of the M phase and slowing declining and reaching low during the anaphase and telophase of M phase. Regulation is achieved by different effector molecules. Phosphorylation of Ki-67 by CDK-1 during M phase leads to the passage through mitosis. CDK 4/6 complex initiate the phosphorylation of retinoblastoma protein (Rb) family in the G1 phase and release transcription factors associated with E2F. As Ki-67 protein is bound to the E2F proteins under cancer conditions upon E2F overexpression, Ki-67 mRNA accumulates.

and ubiquitination ensure balance in cell cycle regulation. For example the protein complex cyclin B/cdc2 kinase are essential regulatory mechanisms that control entry into mitosis. Phosphorylation of Ki-67 by CDK-1 during mitosis leads to the passage through mitosis until metaphase to anaphase transition wherein initiation of sister chromatid separation and reassembly of nucleus take place [31] (Fig. 2). Phosphorylation of the Ki-67 is mediated by multiple kinases which associate and involve in regulation. Ki-67 belong to a family of MPM-2 reactive phosphoproteins comprising structural and functional proteins which are necessary for the control and timing of mitosis [31]. Regulation of MPM-2 is promoted by CDK1 kinase, CDK1 phosphatase, wee1 kinase and M-phase-promoting kinase nimA [34–36]. Other MPM-2 antigens include cdc25, which is a regulator of CDK1 activity at the onset of mitosis [37], DNA topoisomerase II [38] and cdc27 along with cdc16, which are regulatory subunits of the anaphase-promoting complex (APC) that promotes the transition from metaphase to anaphase [39].

On the other hand Cyclin D-dependent kinases (CDK4 and CDK6) have predominant role in the mammalian cell proliferation which help the cell progression to progress into the S phase of the cell cycle via retinoblastoma protein. Under normal conditions CDK4/6 form complex with cyclin D and initiate the phosphorylation of retinoblastoma protein (Rb) family in the G1 phase and exist in the hypophosphorylated form (functionally active form). This leads to the release of transcription factors associated with E2F which drive the expression of E2F responsive genes needed for cell cycle progression. In the late G1 phase Cdk2 binds to cyclin E and complete the phosphorylation of Rb leading to further activation of E2F mediated transcription. This then leads to passage through the restriction point at the boundry of G1/S phase and to initiation of S phase [40]. As cells enter S phase of the cell cycle Rb becomes hyperphosphorylated and remain in this state throughout cell cycle. Phosphorylated Rb has a negative regulatory effect on gene expression by forming complex with E2F [41]. Ki-67 protein is bound to the E2F proteins. It is speculated that under cancer conditions upon E2F overexpression, Ki-67 mRNA accumulates. Since Rb expression is lost in many cancers Ki-67 expression may be under expressed or over expressed. These low and high levels of Ki-67 may be scored as positive to determine the Ki-67 labelling index. This is because only extremely low levels of Ki-67 levels can be detected in the G0 phase or quiescent cells. Whereas in the senescent cells, there is no Ki-67 but some low quantity of Ki-67 staining is present in cells that has stopped proliferating and entered quiescence [4].

Different domains of Ki-67 has important regulatory functions linked with Ki-67 expression. The domain PPD1 binds protein phosphatase 1 (PP1) in Ki-67 is a serine/threonine specific phosphatase involved in mitotic exit by interacting with mitotic kinases [42]. Among different isoforms of PP1, PP1γ has prominent role in chromatin decondensation along nuclear envelope assembly during early anaphase [43]. Studies have shown that PP1γ is required for dephosphorylation of Ki-67 during anaphase. This dephosphorylated state is essential for the anaphase to telophase transition and also other biological activities to target the chromosomes [44]. The FHA domain present in the Ki-67 aids interaction with the phosphorylated proteins such as Hklp2 and hNIKF [33,45]. These proteins has been found to be phosphorylated in the mitotic cells. The exact binding region of FHA with hNIKF is characterised by 44 amino acid residue phosphopeptide corresponding to the residues between 226 and 269 [46]. Hklp2 is a motor protein and are required for mitotic progression.

Detailed characterisation of the promoter region of Ki-67 shows that it lacks TATA box but has GC-rich region which can bind Specificity protein 1 (Sp1), Nuclear factor-kappa B (NF-Kb), cAMP response element binding protein (CREB), E2F transcription factor (E2F), early growth response 1 (EGR-1), Neuron-restrictive silencer element (NRSF), Peroxisome proliferator-activated receptor (PPAR), Maf oncoprotein (Maf) and Wilms' tumour suppressor protein WT1 (WT1). Regulation of Ki-67 gene was thought to be controlled by several factors of which Sp1 plays a crucial role in regulation of basal Ki-67 promoter activity as revealed by electrophoretic mobility shift assay [14,47]. The mechanism of regulation of Ki-67 levels may be thought to be controlled by tumour suppressor p53. Experiments with HeLa cells revealed that p53 inhibited Ki-67 expression in a dose dependent manner. P53 mediate transcriptional repression of Ki-67 by interaction with the p53 binding motif and Sp1 binding sites present in the promoter of Ki-67 [48].

3. Role of Ki-67 as proliferation marker in renal tumorigenesis

The general procedure for determining Ki-67 labelling index is based on performing immunohistochemistry with renal biopsies embedded on paraffin blocks. Generally mouse monoclonal antibody, MIB-1 is used against human Ki-67. Images of the immunostained sections will be visualized under the microscope and data will be processed using the image analysis software [49]. Ki-67 is scored based on the percentage of total number of tumour cells with nuclear staining [30]. Ki-67 labelling index could be used to ascertain grading, stage and several other pathological conditions associated with renal cancer.

Wilms tumour which arise from metanephric blastemal cells is known to be associated with chromosomal abnormalities [50]. Use of prognostic immunohistochemical markers Ki-67 along with tumour suppressor p53 provide information about tumour histology and staging [51,52]. Studies have shown that Ki-67 serve as an independent prognostic marker for renal cancer or could be used in combination with other markers such as MCM-2 [53], survivin, B7-H1 [54], Geminin [55], carbonic anhydrase IX [56], gelsolin [57], MIB-1 [58], phosphorylated S6 protein (pS6) [59] and Vimentin [60]. Ki-67 expression could also be correlated to the mode of surgical treatment and

prognosis. A study has shown that among 71 patients, 16 (22.5%) underwent complete removal of distant metastasis after nephrectomy and 55 (77.5%) underwent cytoreductive nephrectomy. Ki-67/Vimentin expression of < 10% showed higher survival rate in patients who underwent metastasectomy after nephrectomy compared to patients with nephrectomy alone ($P = .001$) [60]. Discrimination between different stages of metastasis like no metastasis, primary metastasis and later metastases could be carried out using Ki-67 marker. Ki-67 cut off of 10% were considered positive in the study. Kankuri et al. [61] have demonstrated that high Ki-67 positivity was observed in primary metastasis patients compared to no metastasis ($p = .007$). Also p53/Ki-67 co-expression was more in primary metastasis patients compared to later metastasis but not observed in no metastasis patients.

4. Prognostic value in renal cancer

Prognosis for cancer has been classically carried out based on tumour size, stage, grade and underlying symptoms. Lack of understanding on the inherent biological heterogeneity of the renal cancers can sometimes cost time and treatment potential of patients with renal cancers. Several studies have investigated the possible involvement of Ki-67 as a prognostic role in renal cancers. Ki-67 act as good molecular surrogate of the aggressive behaviour exhibited by tumours and therapy response for survival outcome assessment in several cancers including RCC. A meta-analysis conducted by Xie and his Colleagues [62] on validating Ki-67 by immunohistochemistry (IHC) expression comprising 20 studies with 5398 patients show that high Ki-67 expression was associated with poor overall survival (OS, Hazard ratio (HR) = 1.95, [95% CI: 1.44–2.64]), cancer specific survival (CSS HR = 1.67, [95% CI: 1.47–1.89]) and disease free survival (DFS, HR = 2.56, [95% CI: 1.79–3.67]). Additionally Ki-67 expression was found to be positively correlated with tumour node and metastasis (TNM) staging (III/IV vs. I/II: RR = 2.03, [95% CI: 1.68–2.44]) pathological T stage (T3/T4 vs. T1/T2: RR = 1.67, [95% CI: 1.35–2.06]), metastasis (yes vs. no: RR = 2.15, [95% CI: 1.77–2.62]), and Fuhrman grade (III/IV vs. I/II: RR = 1.77, [95% CI: 1.20–2.60]). Therefore the researchers concluded that Ki-67 could potentially serve as a biomarker for risk stratification and even as a therapeutic targets in renal cell carcinoma. Another meta-analysis data comprising 4579 patients with 23 studies carried out showed that Ki-67/MIB-1 expression is associated with poor overall survival (OS, Hazard ratio (HR) = 2.06, [95% CI: 1.64–2.57]) and cancer specific survival (CSS HR = 2.01, [95% CI: 1.66–2.44]). Also Ki-67/MIB-1 is correlated with the TNM stage (III/IV vs. I/II: OR = 1.92, [95% CI: 1.61–2.28]), pathological T stage (pT3/T4 vs. pT1/T2: OR = 1.56, [95% CI: 1.21–2.02]), metastasis (M1 vs M0: OR = 1.81, [95% CI: 1.34–2.43] and Fuhrman grade (III/IV vs. I/II: OR = 1.77, [95% CI: 1.21–3.10]). Altogether high Ki-67/MIB-1 expression is correlated with poor prognosis in renal cell carcinoma subjects [58].

The prognostic relevance of Ki-67 as an independent predictor in patients with non-metastatic localized clear-cell renal cell carcinoma was conducted by Gayed et al. [63]. Out of 401 patients studied high expression of Ki-67 was statistically significant to DFS (HR = 3.77, [95% CI: 1.35–10.52, $p < .011$]) but not CSS (HR = 3.51, [95% CI: 0.67–18.35, $p < .137$]. However renal cell carcinoma patients with high expression of Ki-67 were found to have 5-year DFS and CSS rates of 67 and 84%, respectively compared to 87 and 95%, respectively in those with normal expression ($p < .001$ and $p < .05$, respectively). Although Ki-67 has been used as standalone marker for indicating prognosis in most of the renal cancer patients, the involvement of other multiple markers along with Ki-67 has been studied by numerous researchers. In order to provide accurate results on the prognosis related to Ki-67 the findings of the related studies has been highlighted in the Table 1.

Table 1
Selected studies of prognostic relevance of Ki-67 in renal cancer.

SNo	Author and year	Specimen type (No of cases	Detection method	Findings
1.	Kim et al., [59]	Paraffin (351)	IHC	Ki-67 was significantly associated with OS (HR, 2.7), CSS (HR, 3.82), and recurrence free survival (RFS) (HR, 4.85)
2.	Mehdi et al. [53]	Paraffin (50)	IHC	Ki-67 and MCM-2 expression were determined. Both Ki-67 and MCM-2 correlated with grade ($p = .00$; Kruskall-Wallis test).
3.	Gayed et al. [63]	Paraffin (401)	IHC	High expression of Ki-67 were found to have 5-year DFS and CSS rates of 67 and 84%, respectively compared to 87 and 95%, respectively in those with normal expression ($P < .001$ and $P < .05$, respectively)
4.	Kankuri et al. [61]	Paraffin (117)	IHC	p53 and Ki-67 expressions are associated with aggressive tumour phenotype ($p = .036$) and decreased survival in metastatic RCC. P53/Ki-67 coexpression indicate high metastasis probability
5.	Dudderidge et al. [55]	Paraffin (176)	IHC	Ki-67 labelling index > 12% is an independent prognostic markers.
6.	Bui et al. [56]	Paraffin (224)	Tissue microarray (IHC)	Multivariate analysis CA IX and Ki-67 were significant predictors of survival with an HR of 1.78 ($p = .014$) and 1.75 ($p = .009$) respectively.
7.	Visapaa et al. [57]	Paraffin (257)	Tissue microarray (IHC)	Ki-67 is one of the most significant predictor of cancer specific survival ($p < .0001$) Grade 2 tumours with high Ki-67 expression and low gelsolin expression indicates poor cancer specific survival ($p = .0507$).
8.	Lecleresq et al. [51]	Paraffin (73)	IHC	Ki-67 index of 20% is an efficient independent predictor of survival of patients ($p < .00001$).
9.	Delahunt et al. [64]	Paraffin (173)	IHC	Ki-67 indices as < 6% or greater than showed significant survival difference complemented by other markers such as PCNA indices, AgNOR scores and tumour dissemination.

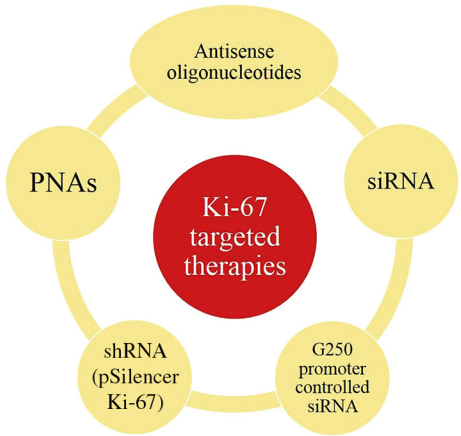

Fig. 3. An overview of treatment strategies employed in preclinical studies targeted against Ki-67. Different approaches have been experimented for decreasing Ki-67 expression in the preclinical studies by different research groups. Different gene silencing technologies like antisense oligonucleotides, Peptide nucleic acids (PNAs), shRNA, siRNA under the control of G250 promoter showed potent decrease of renal cancers with decreased cell proliferation and induced apoptosis.

5. Therapeutic implications of Ki-67 in renal cancer

Treatment of renal cell carcinoma in the advanced stage is a problem of great concern as they are resistant to chemotherapy and radiotherapy [65]. Treatment strategies involving proliferation marker Ki-67 are still in preclinical stages but has shown promising results in both cell culture and mice models of renal cancer (Fig. 3). Deciphering the molecular network associated with the progression of the tumours in kidney would provide solid framework for development of novel treatment options based on Ki-67 for use in clinical trials in the near future. A summary of the therapeutic application has been listed in Table 2.

Some of the preclinical studies carried out for targeting Ki-67 has shown efficacy in both in vitro and in vivo. Studies by Kausch et al. [66] has shown that the efficacy of antisense oligonucleotides for targeting Ki-67 in SK-RC-35 (human renal cell carcinoma) spheroid cultures as well as SCID mice bearing subcutaneous SK-RC-35. Antisense oligonucleotides are synthetic, single stranded nucleic acids which hybridise with complementary target mRNA and hence alter the protein expression either by RNase H mediated degradation or by steric hindrance [67,68]. Researchers claim that oligonucleotide uptake and accumulation is high in the kidney hence serve as a strong determinant for treatment with antisense oligonucleotides. IHC analysis showed potent inhibition of Ki-67 -positive cells (27.8%) in treatment group as compared to control (42.5–57%) and decreased tumour growth was observed in SCID mice ($p = .009$) against systemic administration. Also antisense oligonucleotide treatment also showed apoptosis of the tumour cells. Similar studies carried out with the same research group using RENCA cells in vitro and also in RENCA cells implanted under the renal capsule of mice showed decrease in Ki-67 levels and cell growth. Studies in vivo showed reduction in syngeneic kidney tumours ($p < .05$). Development of lung metastases was also significantly reduced in antisense oligonucleotide treated animals (10%) compared to control (30–40%) [69]. Derivatives of antisense oligonucleotides, Peptide nucleic acids (PNAs) has also been tested by research groups. PNAs are generally DNA mimic which consist of aminoethyl glycine units instead of deoxyribose-phosphate backbone which can bind to complementary DNA and RNA sequences and inhibit replication and transcription [70]. A study by Zheng et al. [71] has shown that PNAs could significantly decrease the proliferation rate in a dose dependent manner and induced apoptosis in the RCC cell line studied.

Table 2
Promising therapeutic application in preclinical studies.

SNo	Author and year	Preclinical/Clinical setting	Treatment method	Comments
1.	Liu et al. [78]	Preclinical (Human renal cell carcinoma cell line and RCC tumour xenograft established BALB/c nude mice)	G250 promoter controlled oncolytic adenovirus expressing Ki-67 siRNA	Result showed G250 inhibited RCC proliferation and induced apoptosis. The oncolytic adenovirus expressing Ki-67-siRNA displayed strong growth inhibition effects on RCC xenografts
2.	Zheng et al. [77]	Preclinical (Human renal cell carcinoma cell line)	Small-interfering RNA (siRNA) targeted against Ki-67	Experiments demonstrated reduction of Ki-67 mRNA expression in Ki-67 siRNAs treated cells. Ki-67 siRNA treatment resulted in inhibition of proliferation and increased apoptotic cell death
3.	Zheng et al. [76]	Preclinical (Human renal cell carcinoma cell line)	Expression plasmid encoding shRNAs against the Ki-67 gene, pSilencerKi-67	pSilencer Ki-67 inhibiting proliferation and inducing apoptotic cell death.
4.	Zheng et al. [71]	Preclinical (RCC 786–0 cells)	Peptide nucleic acids (PNA) against Ki-67	Anti-Ki-67 PNA has strong and dose dependent effects on proliferation and apoptosis of human RCC cell lines
5.	Kausch et al. [66]	Preclinical (3D RCC spheroid cultures and RCC SCID mouse model)	Antisense oligonucleotides (asON) directed against Ki-67	IHC analysis showed potent inhibition of Ki-67 –positive cells (27.8%) in treatment group as compared to control (42.5–57%)
6.	Kausch et al. [69]	Preclinical (Murine RENCA cells and RENCA implanted Balb/c mice)	Antisense oligonucleotides (asON) directed against Ki-67	Ki-67-directed antisense oligonucleotides inhibited target protein expression and proliferation of tumour cells in vitro and tumour growth ($p < .05$) and lung metastasis formation (10%) in murine renal cell carcinoma

Over the last few years, newer modes of regulation of gene expression and their application in cancer therapy are increasing. Several different mechanism include DNA plasmid coding to shRNA, small hairpin RNA (shRNA; a single strand RNA with a hairpin loop) and small-interference RNA (siRNA; a double strand RNA) [72]. siRNA are usually of 21–25 nucleotides length ds RNA incorporated into the RNA-induced silencing complex (RISC) where they initiate the degradation of the target mRNA in sequence specific manner. Alternatively shRNA may be transcribed by RNA pol III or modified pol II promoters transfected as plasmid vectors. Unlike siRNA, wherein the duplex is directly delivered to the cytosol shRNA are capable of integrating with DNA and consist of two complementary 19 to 22 bp RNA sequences linked by a short loop of 4–11 nucleotides. After transcription the shRNA sequence is transported to the cytosol where it is recognised by DICER which processes the shRNA into the siRNA duplexes. Like exogenously delivered synthetic siRNA, these endogenously processed siRNA incorporated into the RNA-induced silencing complex (RISC) where they initiate the degradation of the target mRNA in sequence specific manner [73]. The advantage of shRNA compared to siRNA is that siRNA are less stable and rapidly degrade in the tumour tissue. However plasmid expressing shRNA are prolonged and result in stable expression of the RNAi effector molecules [74,75]. A study conducted by Zheng et al. [76] has shown that expression plasmid encoding shRNA against Ki-67, pSilencerKi-67 when transfected into human renal carcinoma cell line inhibited the proliferation and induced apoptosis. Another study by the same research group employing siRNA has shown that reduction of Ki-67 mRNA expression in Ki-67 siRNAs treated cells by RT-PCR analysis and in-situ hybridisation. Ki-67 siRNA treatment resulted in inhibition of proliferation and increased apoptotic cell death in 786–0 renal carcinoma cell line [77]. Liu et al. [78] have demonstrated that oncolytic virus, whose early virus genes for replication such as E1A gene transactivate adenoviral genes is under the control of a renal cell carcinoma specific promoter – the G250 promoter. G-250, a cell surface antigen is expressed on 90% of renal cancer cells and 82% metastatic renal cancer lesion but not in the normal kidney cells. The constructed oncolytic virus G250-Ki-67 is armed with transgene of Ki-67-siRNA. Results show that the expression of Ki-67 gene in 786–0 cell lines were suppressed by these adenoviruses. Altogether G250 promoter regulated adenovirus could highly amplify and express Ki-67-siRNA in renal cancer cells with expression of G250 antigen, inhibit renal cancer cells proliferation and induce apoptosis and potent growth inhibition effects on RCC xenografts in nude mice.

6. Conclusion

Substantial evidences highlight the importance of molecular proliferation marker Ki-67 as a potent tool for prognosis and treatment in renal cancer. More robust data on prognostic potential of Ki-67 have revolutionised the treatment options in renal cancer and improved the clinical outcome in patients. Therapies targeting Ki-67 is still in the preclinical stage with more detailed strategical studies required for extrapolation to the clinical use. Current understanding of the molecular networks associated and precise role of Ki-67 could streamline the basis of cancer progression and metastasis in renal cancer patients, thus heralding the possibility of early diagnosis and treatment.

Conflicts of interest

There are no conflicts of interest.

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