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DNA-PK in human malignant disorders: Mechanisms and implications for pharmacological interventions

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ABSTRACT

The DNA-PK holoenzyme is a fundamental element of the DNA damage response machinery (DDR), which is responsible for cellular genomic stability. Consequently, and predictably, over the last decades since its identification and characterization, numerous pre-clinical and clinical studies reported observations correlating aberrant DNA-PK status and activity with cancer onset, progression and responses to therapeutic modalities. Notably, various studies have established in recent years the role of DNA-PK outside the DDR network, corroborating its role as a pleiotropic complex involved in transcriptional programs that operate biologic processes as epithelial to mesenchymal transition (EMT), hypoxia, metabolism, nuclear receptors signaling and inflammatory responses. In particular tumor entities as prostate cancer, immense research efforts assisted mapping and describing the overall signaling networks regulated by DNA-PK that control metastasis and tumor progression. Correspondingly, DNA-PK emerges as an obvious therapeutic target in cancer and data pertaining to various pharmacological approaches have been published, largely in context of combination with DNA-damaging agents (DDAs) that act by inflicting DNA double strand breaks (DSBs). Currently, new generation inhibitors are tested in clinical trials. Several excellent reviews have been published in recent years covering the biology of DNA-PK and its role in cancer. In the current article we are aiming to systematically describe the main findings on DNA-PK signaling in major cancer types, focusing on both preclinical and clinical reports and present a detailed current status of the DNA-PK inhibitors repertoire.

Contents

Abbreviations: AMPK, AMP-activated protein kinase; AR, androgen receptor; ATM, ataxia-telangiectasia mutated; ATR, ATM and Rad3-related; BER, base excision repair; CHK1, checkpoint kinase 1; CHK2, checkpoint kinase 2; CK2, casein kinase 2; CLL, chronic lymphocytic leukemia; CNV, copy number variation; CR, complete response; CRC, colorectal cancer; DDAs, DNA-damaging agents; DDR, DNA damage response; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; ds, double-stranded; DSB, double-strand break; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; HCC, hepatocellular carcinoma; HR, homologous recombination; IR, ionizing radiation; MMR, mismatch repair; MSI, microsatellite instability; NHEJ, non-homologous end-joining; NSCLC, non-small cell lung cancer; OS, overall survival; PIKK, phosphatidylinositol-3-kinase-related protein kinase family; PLD, pegylated liposomal doxorubicin; PR, partial response; RCC, renal cell carcinoma; RT, radiation therapy; SCID, severe combined immune-deficiency; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; SSB, single-strand break; TCGA, The Cancer Genome Atlas; UM, uveal melanoma.

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1. Introduction

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs), encoded by the *PRKDC*/*XRCC7* gene, is a pivotal DNA damage response (DDR) player and with 460kD also the largest member of the phosphatidylinositol-3-kinase-related protein kinase family (PIKK) that includes also the DDR master kinases ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia mutated and Rad3-related (ATR) (Blackford and Jackson, 2017). Apart of the 3 DDR kinases, the PIKK family includes also mTOR, SMG1 and TRRAP (Lempiainen and Halazonetis, 2009).

Together with the Ku heterodimer that consists of the Ku70 (XRCC6) and Ku80 (XRCC5) subunits, DNA-PKcs forms the DNA-PK holoenzyme complex that is the core component of the non-homologous end-joining (NHEJ) machinery, which alongside homologous recombination (HR), comprise the two major canonical pathways for DNA double strand breaks (DSBs) repair.

NHEJ entails several steps that cover the detection of DSBs by the Ku70/80 ring-shaped heterodimer with subsequent recruitment, assembly and stabilization of the NHEJ complex at the damage location and finally, the enzymatic activation of DNA-PKcs, which leads eventually to ligation of broken DNA ends (Davis and Chen, 2013). While the homologous template-dependent HR is largely restricted to the S and G2 cell cycle phases, NHEJ is essentially active during G0 and G1 phases, however, it may still be functional through all cell phases (Trenner and Sartori, 2019). It has been suggested for example that pathway choice between HR and NHEJ during S phase is regulated through an interaction between the tumor suppressor protein BRCA1 and the DNA-PK regulatory subunit Ku80, which stabilizes the Ku heterodimer at DSBs, a step that is required for end-joining repair through NHEJ in the G1 phase (Saha and Davis, 2016). As aforementioned, at occurrence of DSBs, DNA-PKcs is rapidly recruited by the Ku70/80 heterodimer to the affected DNA lesions, at which two units of DNA-PKcs interact with two heterodimers of Ku70/80 to assemble the DNA-PK complex (Grundy et al., 2014). The formation of the holoenzyme on DNA stimulates the catalytic activity of DNA-PKcs, which is manifested by its autophosphorylation and a subsequent phosphorylation of NHEJ effectors including Ku70, Ku80, the scaffold proteins XRCC4 and XLF, the structure-specific endonuclease Artemis, and the polynucleotide kinase/phosphatase PNKP (Blackford and Jackson, 2017; Davis et al., 2014). The autophosphorylations of DNA-PKcs on Thr2609/2647 and Ser2056, in the ABCDE and PQR clusters, respectively, are involved in regulation of its DNA end-processing DSBs repair activity (Cui et al., 2005; Jiang et al., 2015). Within its function in the DDR and apart of being a critical mediator of cellular response to constantly occurring DSBs, DNA-PKcs is responsible for the rejoining of programmed DSBs that are generated during V(*D*)J recombination and class switching recombination in the course of B and T lymphocytes maturation (Chang et al., 2017; Franco et al., 2008). Additionally, the NHEJ is the predominant pathway for DSBs resulting from exposure to ionizing radiation (IR) and it is considered to be responsible for the rapid repair of up to 85% of IR-induced DSBs (Mahaney et al., 2009).

The very high cellular levels of DNA-PK (Anderson and Carter, 1996) have suggested that apart of NHEJ, this complex may be involved also in global pathways that are not linked to DNA repair. Indeed, and aside of its well-characterized DDR-associated functions, the DNA-PK complex has also been shown to have a hold over multiple cellular processes including transcriptional regulation. In fact, one of the first studies to reveal DNA-PK's biologic function demonstrated a DNA-PK-regulated phosphorylation of the SP1 transcription factor upon its binding to GC transcriptional regulatory elements in the context of SV40 early genes transcription (Jackson et al., 1990). Shortly then after, Dvir et al. reported in 1992 that DNA-PK regulates RNA polymerase II through phosphorylation in the presence of DNA and the TFIID, TFIIB, and TFIIF transcription factors (Dvir et al., 1992). During the last two decades, a myriad of findings (reviewed in (Goodwin and Knudsen, 2014)) have linked DNA-PK to regulation of transcription of various cellular networks that control among others expression of immune effectors, tumor suppressors and hormone receptors such as the androgen and estrogen receptors that play critical roles in human cancer (Goodwin and Knudsen, 2014). Additionally, DNA-PK can directly phosphorylate the glucocorticoid and the progesterone receptors (Giffin et al., 1997; Sartorius et al., 2000).

In recent years, DNA-PK has been also correlated with key metabolic processes such as fatty acids synthesis and glucose metabolism (Wang et al., 2015; Wong et al., 2009), reviewed in (Chung, 2018). In that respect, Wong et al. suggested already back in 2009 that DNA-PK regulates insulin-associated transcription of fatty acid synthase, a key lipogenesis player (Wong et al., 2009). Later, the inhibition of DNA-PK has been linked to improved insulin sensitivity and to protection against type 2 diabetes through its regulation of phosphorylation of AMP-activated protein kinase (AMPK) and increased mitochondrial function (Park et al., 2017). Among other pleiotropic non-DDR cellular functions of DNA-PK, the most notable are its involvement in the protection of telomeres length (Bailey et al., 1999; Williams et al., 2009), regulation of mitosis (Jette and Lees-Miller, 2015), for example through its interaction with polo-like kinase 1 (Douglas et al., 2014), and functional control of cytoplasmic organelles such as the Golgi apparatus (Farber-Katz et al., 2014). Recently, a manuscript by Shao et al. provided evidence that DNA-PKcs has a DNA repair-independent, but crucial role in ribosomal RNA biogenesis, which is primarily dependent on Thr2609 cluster phosphorylation (Shao et al., 2020). Interference with Thr2609 phosphorylation resulted in global protein synthesis deregulation and eventual bone marrow failure (Shao et al., 2020).

Anomalous DDR signaling, affecting either cell cycle checkpoints or DNA repair elements, has been firmly associated with cancer propensity and may alter tumor cell responses to DNA-damaging agents, largely used in clinical oncology. Therefore, DDR pathways make an ideal target for therapeutic intervention. As several reviews in recent years have covered overall aspects of DNA-PK biology under physiologic and deregulated conditions, here we aim to systematically overview and discuss pre-clinical and clinical findings pertaining to the role of the DNA-PK system across common human cancer types.

2. DNA-PK in cancer – General aspects of preclinical observations

Initial observations evidencing that aberrant NHEJ signaling leads to genomic instability and eventual carcinogenesis originated largely from genetically-modified animal-based studies that generated mice with DNA-PK ablations (reviewed in (Mohiuddin and Kang, 2019)). The early studies assessing biologic consequences of a compromised NHEJ have focused on the impact of Ku70- and Ku80-deficiency on cellular models and mice. Overall, Ku70- and Ku80-deficient cells and mice were shown to exhibit radiation hypersensitivity and defective NHEJ with associated obvious genomic instability features as chromosomal aberrations and aneuploidy (S. Chen et al., 2001; Difilippantonio et al., 2000; Ferguson et al., 2000; Gu et al., 1997a, 1997b; Lee et al., 2016; Nussenzweig et al., 1997). As might be envisaged with a faulty NHEJ pathway, Ku-deficient animals have aberrant V(*D*)J recombination with resultant abnormalities in the maturation processes of both B and T lymphocytes (Gu et al., 1997a, 1997b; Li et al., 1998). Li et al. reported in late 1990's that mice with Ku70 depletion develop T cell lymphoma (Li et al., 1998) while mice depleted of Ku80 on a deficient p53 background develop at young age a lymphocytic malignancy that exhibits features similar to Burkitt's lymphoma (Difilippantonio et al., 2000). With respect to DNA-PKcs, the severe combined immunedeficiency (SCID) mouse (Bosma et al., 1983) harbors a spontaneously occurring mutation in the *PRKDC* gene (Finnie et al., 1995; Kirchgessner et al., 1995; Peterson et al., 1995), leading to an 83-aa truncation of the C-terminal end of the protein (Blunt et al., 1996; Danska et al., 1996). Consequently, DNA-PKcs levels in these mice are only about 10% compared to wild type animals, and DNA-PK activity is

considerably reduced (Danska et al., 1996; Woo et al., 1998). SCID mice are highly radiosensitive and exhibit profound T and B cell deficiency due to a defective V(*D*)J. Additionally, the animals present with shortened telomeres and a subsequent accelerated aging.

Among the first works to assess the impact of DNA-PKcs deficiency in carcinogenesis was a study by the group of Gloria Li who reported in 1999 that mice with a DNA-PKcs disruption demonstrate a predisposition for the development of hyperplastic polyps and abnormal crypt foci in the intestine (Kurimasa et al., 1999). A complementary later study by Espejel et al. reported that DNA-PKcs-ablated aged mice showed increased incidence of thymic lymphomas in addition to other features including short telomeres, smaller body size and overall earlier onset of age-related pathologies (Espejel et al., 2004). In addition to these findings, naturally occurring mutations in *PRKDC* in BALB/C mice, affecting codons 2140 (R2140C) and 3844 (M3844V), have been correlated with increased susceptibility to IR-induced genomic instability and mammary epithelial cells transformation (Yu et al., 2001). It has been previously reported that IR-induced DNA-PKcs activation through phosphorylation at the Thr2609 cluster is predominantly regulated by ATM (B. P. Chen et al., 2007). Mechanistically, it was shown that ablation of DNA-PKcs Thr2609 cluster phosphorylation results in a considerable increase in basal and radiation-induced telomere fusions (Williams et al., 2009). Homozygous knock-in mice harboring threonine to alanine substitutions at the Thr2605 cluster (Thr2605A, Thr2634A and Thr2643A) die shortly after birth due to bone marrow failure and this severe phenotype manifestation can be rescued through bone marrow transplantation (Zhang et al., 2011). Interestingly, in 2016 Zhang et al. reported that mice with loss-of-function phosphorylation at DNA-PKcs cluster Thr2605 develop a range of tumors including lymphomas, hepatocellular carcinoma and squamous cell carcinoma as of 15 weeks of age after being rescued through bone marrow transplantation (Zhang et al., 2016).

In addition to the studies focusing on the correlation between deregulated DNA-PK and cancer, similar observations within the context of pre-clinical models have been reported and linked loss-of-function of other NHEJ players as LIG4, XLF and XRCC4, mainly on a p53-null background, to the development of particular tumors such as sarcoma (Sharpless et al., 2001), B-cell lymphoma (Gao et al., 2000) and medulloblastoma (G. Li et al., 2008; C. T. Yan et al., 2006).

3. DNA-PK expression and signaling in human malignancies

An immense body of experimental data has been collected over the years on the roles of DNA-PK in human cancer. The data that corelate alterations as CNVs (Fig. 1), expression patterns (Fig. 2) and signaling of DNA-PK components with tumor onset, progression and responses to therapies, may be distinct and vary in individual malignancies. In this chapter we review cancer types-specific peculiarities with respect to expression, SNPs (Table 1), downstream signaling and the pleiotropic interactions of the DNA-PK complex with other cellular networks (Fig. 3). Additionally, studies referring to DNA-PK-related interference with anti-cancer therapeutic modalities and to DNA-PK targeting in context of different tumor types are discussed.

3.1. Breast cancer

A first significant observation correlating a DNA-PK genetic aberration with breast tumorigenesis was reported by the 2001 Yu et al. study that described two different *PRKDC* single nucleotide polymorphisms (SNPs) as a basis for a high-risk breast carcinogenesis in BALB/C mice following exposure to IR (Yu et al., 2001). A later work by Fabre et al. suggested that this polymorphism is functionally underlying a deficiency of DNA-PKcs expression with consequent compromising effects on DNA repair and telomere function (Fabre et al., 2011). Respectively, polymorphisms in the promoters of the Ku70 and Ku80 subunits of DNA-PK have been correlated with either increased or decreased risk in several human tumors, including breast cancer. Accordingly, both the Ku70 C-61G polymorphism (SNP rs2267437) and the Ku80 promoter polymorphism G-1401 T (SNP rs828907) were associated with an elevated risk for the development of breast cancer (Willems et al., 2009). Conversely, the *XRCC6* A46922G SNP rs132793 variant was suggested to relate to a decreased breast cancer risk (Jia et al., 2015; Sobczuk et al., 2010). Mechanistically, it is to be anticipated that at least in some of SNPs, mutations in promoters of DNA-PK components interfere with transcription regulation, thereby affecting eventually NHEJ overall functioning. A confirmation for that was provided by a study by Quimet et al. who found that the Ku70 C-61G variant (rs2267437C > G) substantially changes transcriptional activity in an allele-specific manner and influences the DNA-protein complex formation in breast cancer cell lines (Ouimet et al., 2012).

A number of studies reported observations pointing to the relevance of DNA-PKcs and the Ku70 and Ku80 subunits expression levels to breast cancer pathogenesis. These findings have associated DNA-PK decreased expression with increased frequency of chromosome instability and consequent clinical parameters as larger tumors, higher tumor grade and axillary lymph node metastasis (Someya et al., 2007). A similar correlation has been observed in tumors with lower Ku70 and Ku80 expression (Someya et al., 2007). Tumors expressing low levels of DNA-PKcs were also associated with worse breast survival regardless the status of the estrogen receptor (Abdel-Fatah et al., 2014a, 2014b). Likewise, DNA-PK lower activity in peripheral blood lymphocytes, which was determined by using a DNA-pull-down assay, was found to correlate with a risk for breast and uterine cervix cancer (Someya et al., 2006).

Apart of the impact of promoter polymorphism, among other potential mechanisms leading to deregulated DNA-PK expression, the CDK12/Cyclin K complex has been reported to affect genome stability through the regulation of RNA polymerase II activity, which controls the transcription of various DDR components, including BRCA1 and FANC1 (Blazek et al., 2011). In 2018, Naidoo et al. reported that CDK12 can be either highly expressed or absent in patient breast cancer samples. Importantly, CDK12 deficiency was associated with reduced expression of several DDR proteins including both Ku70/Ku80 and DNA-PKcs (Naidoo et al., 2018). These findings advocate a novel mechanism of CDK12 in DNA-PK dysregulation in breast cancer, with possible future therapeutic implications.

In another work, Li et al. reported nuclear bFGF to mediate chemotherapy resistance of triple-negative breast cancer (Li et al., 2015). The study showed that chemo-residual triple-negative tumor cells with increased bFGF nuclear presence manifest also elevated levels of both DNA-PKcs and Ser2056 phosphorylated, activated DNA-PKcs. Furthermore, high nuclear bFGF localization was associated with an elevated DNA-PK-based DNA damage repair activity.

With respect to therapeutic responses, a 2010 study by Söderlund Leifler et al. that correlated DNA-PK levels in a cohort of 224 breast cancer patients with response to radiotherapy, reported that patients with lower Ku70/Ku80 levels had better outcome at early stages of the disease (Soderlund Leifler et al., 2010). Similar observations in the same study were found for patients with higher levels of DNA-PKcs. Additionally, high nuclear Ku70/80 levels were correlated in breast cancer with various parameters of poor prognosis as higher histopathological grade, lymphvascular invasion, absence of estrogen receptor and a basal-like phenotype (Alshareeda et al., 2013). Similarly, the Ku70/80 heterodimer was shown to be expressed and bound to chromatin at elevated levels in advanced breast cancer compared to hyperplastic and normal breast cells (Abdelbaqi et al., 2013).

Various DNA-PK-related mechanisms that may affect treatment modalities have been described in breast cancer (Lees-Miller et al., 2016; Medunjanin et al., 2010; Rubin et al., 2007; Ter Brugge et al., 2016). For example, Ku70, Ku80 and DNA-PKcs were identified as HOXB7-binding proteins, an interaction, which may contribute to IR resistance in mammary epithelial cells due to an enhanced NHEJ activity (Rubin et al., 2007). Interestingly, two estrogen receptor-alpha (ER-alpha)-

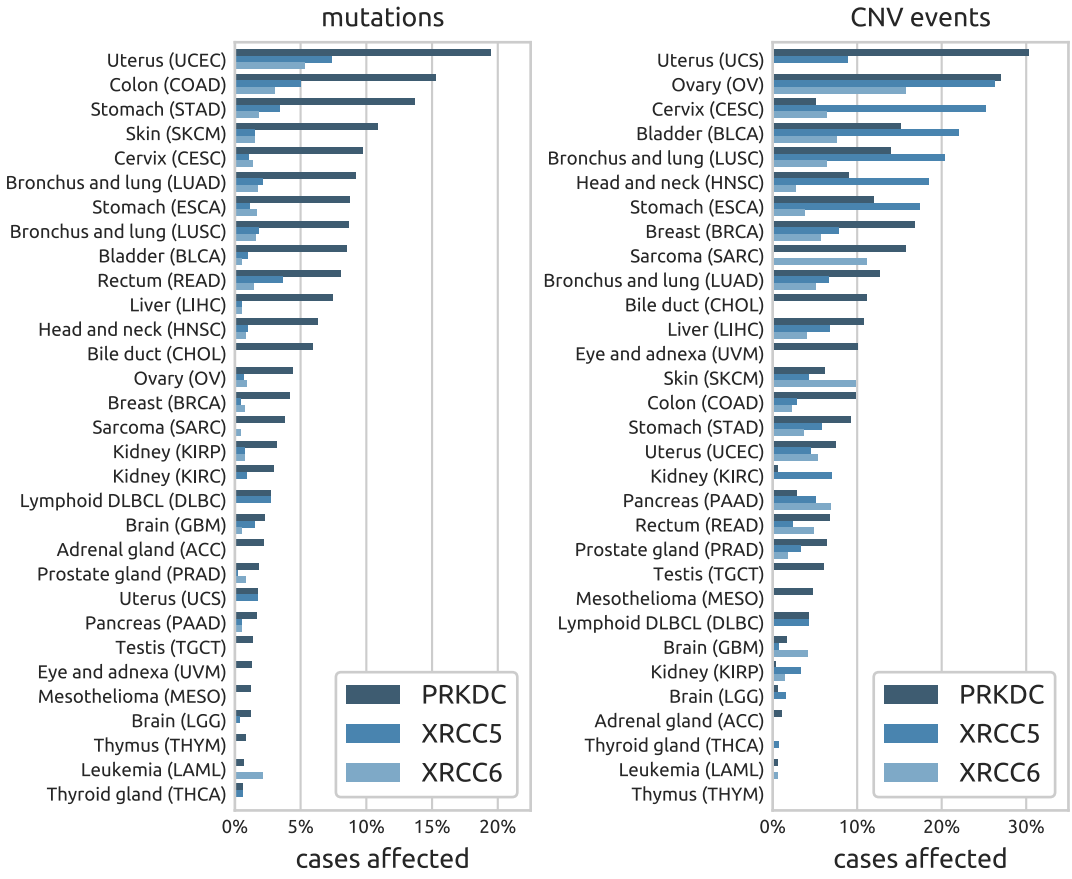

Fig. 1. The fraction of cases with a mutation (left) or CNV event (right) for the *PRKDC*, *XRCC5* and *XRCC6* genes in individual TCGA projects on various cancer types. The data has been obtained from the GDC Data Portal (https://portal.gdc.cancer.gov/genes/ENSG00000253729 for PRKDC, for example). More details on the UCEC project, for example, can be obtained at https://portal.gdc.cancer.gov/projects/TCGA-UCEC.

binding sites upstream of the DNA-PKcs initiation site have been described and reported to enhance breast cancer cells to repair DNA damage, which could lead to therapy resistance (Medunjanin et al., 2010). Likewise, the long non-coding RNA LINP1 has been suggested as a scaffold, which links Ku70/80 and DNA-PK, allowing an efficient DSBs repair through the NHEJ pathway and contributing to the resistance of breast cancer to IR and chemotherapy (Lees-Miller et al., 2016).

On the other hand, an interference with DNA-PK function by a truncated RAF1 splice variant, Raf1-tr, which shows increased nuclear localization and binds DNA-PK, sensitizes various cancer cells, including those of breast origin, towards IR and the radiomimetic bleomycin (Nixon et al., 2019). Different new DNA-PK-based strategies have been studied to sensitize breast cancer cells to IR and chemotherapeutic drugs. For example, the peptide HNI-38 inhibits DNA-PK activity through the disruption of the interaction between the Ku heterodimer and DNA-PKcs (Kim et al., 2002). Also, the DNA-PK inhibitors NU7441 and KU-55933 showed sensitization to IR and doxorubicin due to the accumulation of DNA damage (Ciszewski et al., 2014; Cowell et al., 2005). Likewise, an increased DNA damage has been observed in BRCA1-BER-deficient cancer cells treated with DNA-PK inhibitors alone or in combination with cisplatin (Albarakati et al., 2015). Interestingly, a study investigating the signaling interphase between EGFR and DNA-PK reported that the EGFR inhibitor gefitinib reduced DNA-PKcs nuclear levels and increased its cytosolic localization, which was correlated to a decreased function of DNA-PKcs (Friedmann et al., 2006). These works reflect the potential significance of DNA-PK targeting in sensitizing breast cancer cells to IR and chemotherapy.

DNA-PK has been also pointed to play an active role in the carcinogenesis of breast cells. Anandi et al. showed in a 2017 study that DNA-PK is in fact driving the transformation process of normal breast cells following treatment by an alkylating agent (Anandi et al., 2017). The authors found that exposure of breast epithelial cells to *N*-methyl-N-nitrosourea resulted in an altered function of the cellular Golgi apparatus that led to an epithelial-to-mesenchymal transition (EMT)-like phenotype and cell transformation. Inhibition of DNA-PK was in turn able to completely reverse the altered Golgi phenotype and to partially rescue the cells from the EMT-like alterations.

Earlier this year, a link between DNA-PKcs and autophagy has been described in MCF-7 breast cancer cells (Puustinen et al., 2020). The provided data suggest that DNA-PKcs mediates the phosphorylation of the AMP-dependent protein kinase (AMPK) subunit PRKAG1/AMPKγ1 that subsequently primes AMPK complex to the lysosomal activation, thereby linking DNA-PK to autophagy and cellular metabolism.

3.2. Cervical cancer

Mutations and CNV alterations in the genes encoding the catalytic and regulatory subunits of DNA-PK are often present in cervical cancer (Fig. 1). Interestingly though, the expression levels of the Ku70 and Ku80 regulatory subunits particularly, seem to correlate most with patients outcome and treatment responses as indicated by various studies. A 2000 report by Wilson et al. on cervical carcinoma biopsies has revealed that tumors with low Ku70 protein expression were more radiosensitive and correlated with a significantly better patient survival (Wilson et al., 2000). Similar results were obtained with respect to the correlation between Ku80 expression and response to radiation therapy and survival. Harima et al. reported in 2003 that a low expression of the Ku80 protein leads to radiosensitivity in cervical cancer patients and

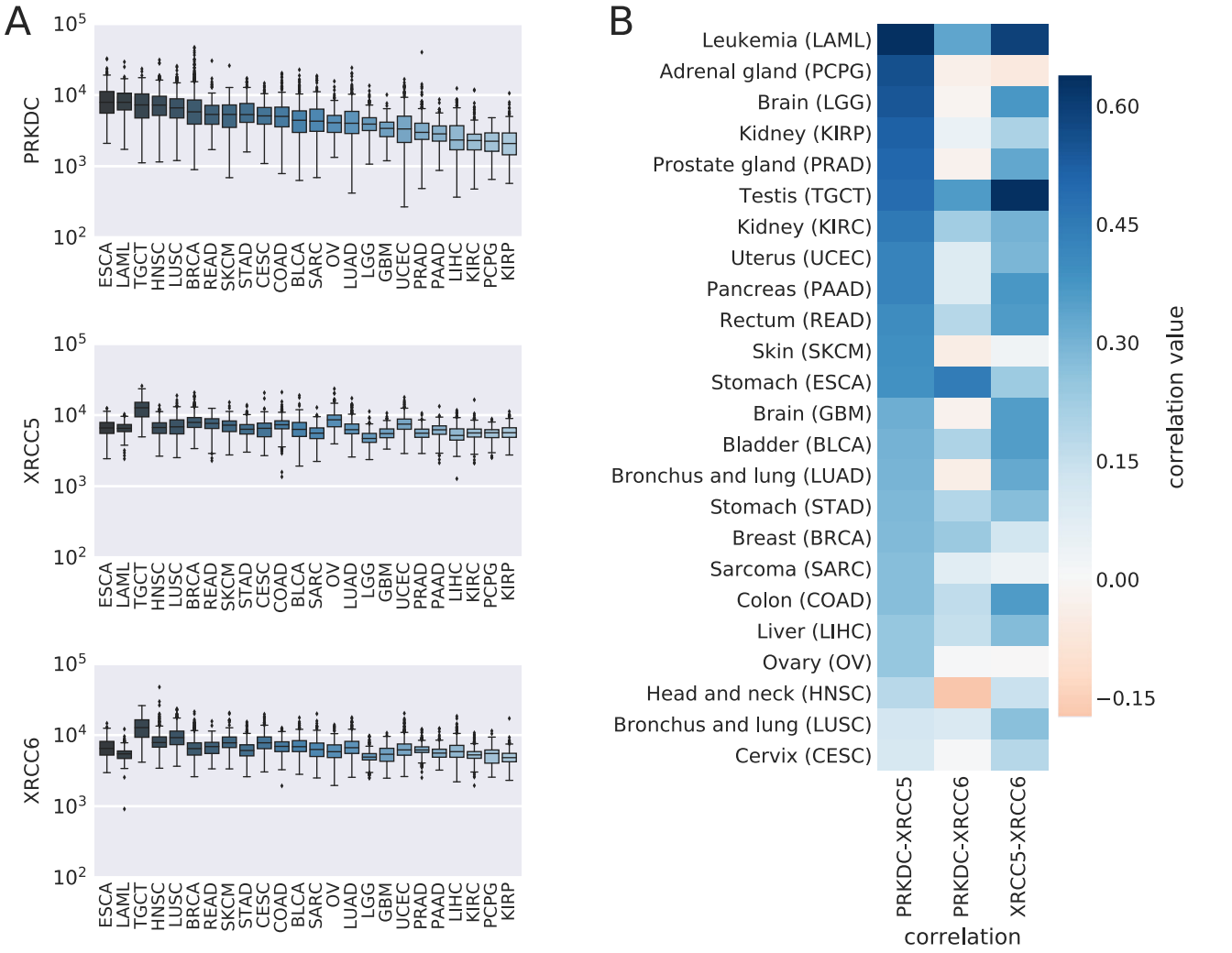

Fig. 2. (A) Distributions of normalized expression values of the *PRKDC*, *XRCC5* and *XRCC6* genes in primary tumor samples (Primary Solid Tumor or Primary Blood Derived Cancer) in individual TCGA projects on various cancer types. Only the projects with at least 100 primary tumor samples are shown. We use here the transcriptomics data made available by the Pan-Cancer Atlas initiative (https://gdc.cancer.gov/about-data/publications/pancanatlas). The Pan-Cancer Atlas initiative compares the 33 tumor types profiled by The Cancer Genome Atlas (TCGA). The expression data use Fragments Per Kilobase of transcript per Million (FPKM) with Upper-Quartile (UQ) normalization and possible batch effects have been removed. (B) Correlations between log-transformed normalized expressions of the *PRKDC*, *XRCC5* and *XRCC6* genes in primary tumor samples (Primary Solid Tumor or Primary Blood Derived Cancer) in individual TCGA projects. Only the projects with at least 100 primary tumor samples are shown.

predicted that Ku80 plays a role in treatment outcome (Harima et al., 2003). Additionally, a study by Saygili et al. suggested that although Ku70 expression may not be a prognostic marker in endometrial carcinoma patients, disease-free survival was found to be significantly higher in patients with low percentage of Ku70-positive tumor cells (Saygili et al., 2004).

Ku80 and Ku70 have been evaluated as potential predictive biomarkers for patients with stage IB-IIA cervical carcinoma treated with preoperative brachytherapy and radical surgery. However, although tumor tissues express these proteins in contrast to normal epithelium, no correlation of their expression with radiation response has been found (Beskow et al., 2006). A subsequent 2009 retrospective study of

Table 1
SNP variants in genes encoding the DNA-PK complex for which correlations with clinical parameters have been reported.

SNP	Gene	Function/Association	Cancer type
rs7003908	*PRKDC*/*XRCC7* (DNA-PKcs G6721T)	• increased cancer risk • splicing regulation, mRNA instability (Sipley, J.D. et al., 1995)	CRC (Sishc and Davis, 2017), prostate (Mandal, Kapoor, & Mittal, 2010), glioma (Wang et al., 2004)
rs8178158	*PRKDC*/*XRCC7* (DNA-PKcs)	• increased cancer risk	metastatic melanoma (Liang et al., 2012)
rs828907	*XRCC5* (Ku80 G-1401 T)	• increased cancer risk	breast (H. C. Wang, et al., 2009), CRC (M. D. Yang et al., 2009b), gastric (J. Q. L, et al., 2011), head and neck (Hsu et al., 2009)
rs132793	*XRCC6* (Ku70 A46922G)	• decreased cancer risk	breast (Jia, Ren, Yan, Xiao, & Sun, 2015; Sobczuk et al., 2010)
rs2267437	*XRCC6* (Ku70 C-61G)	• increased cancer risk • affects transcriptional activity (Ouimet et al., 2012)	breast (Willems et al., 2009)
rs5751129	*XRCC6* (Ku70 T-991C)	• increased cancer risk • low mRNA and protein expression in NPC (Huang et al., 2015)	CRC, head and neck, gastric cancer (M. D. Yang, Wang, Chang, Tsai, & Bau, 2011)

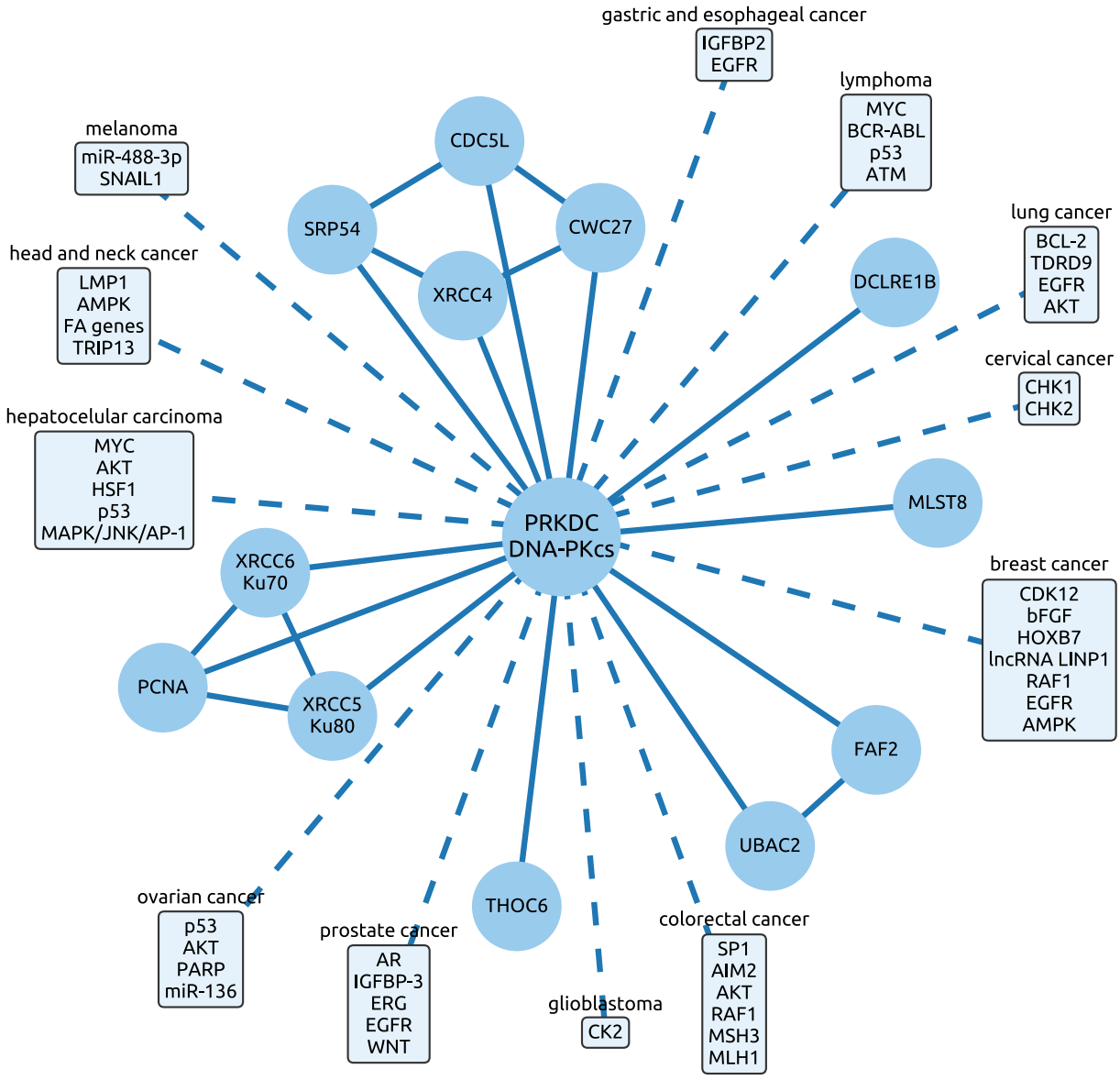

Fig. 3. DNA-PKcs-interacting proteins and its associated partners. The network composed of blue circles and full edges shows the DNA-PKcs interactome based on the STRING database data (www.string-db.org), considering only experimentally confirmed interactors with high (0.700) confidence. Dashed edges lead to DNA-PKcs interactors and DNA-PKcs-associated proteins, miRNAs or long non-coding RNA (lncRNA) especially relevant to particular cancer types; they are based on the findings of the literature included in this review. The exact nature of their associations with DNA-PKcs is in detail described in the corresponding review sections.

IB-IIA cervical cancer patients receiving preoperative radiotherapy followed by radical surgery has shown increased frequency of DNA-PKcs-, Ku70- and Ku80-positive cells in residual tumors as compared to the corresponding primary cancers, indicating a possible radioresistance mechanism (Beskow et al., 2009). Another prognostic study for locally advanced cervical cancer treated with cisplatin-based chemoradiotherapy (CRT) found a marginal significance for 5-year survival difference between low and high expressors of DNA-PKcs (30% *vs.* 60%, $p = .05$) (Ho et al., 2017).

With respect to interference with the NHEJ pathway in cervical cancer, only a few of preclinical studies utilizing simple models have been performed. In this respect, Tian et al. have reported back in 2007 that the downregulation of DNA-PKcs and Ku70 using siRNAs in HeLa cells leads to a decreased proliferation and increased apoptosis upon treatment with DDP (Tian et al., 2007). More recently, Vávrová et al. demonstrated that DNA-PK inhibition by NU7441 in HeLa cells increases phosphorylation of CHK1 and CHK2 upon a single dose irradiation of 8Gy, which contrasted with a complete blockade of CHK2 Thr68 and CHK1 Ser345 phosphorylations upon treatment with the ATM kinase inhibitor KU55933 and the ATR inhibitor VE-821, respectively (Vavrova et al., 2016). NU7441 also mediated a strong G2 arrest 24 h post 15Gy of IR that was coupled to the radiosensitizing effect of NU7441 only 72 h after irradiation, contrasting with the VE-821-based radiosensitization observed shortly post irradiation (Vavrova et al., 2016). Further studies testing novel DNA-PK inhibitors in a broad spectrum of *in vitro* and *in vivo* cervical cancer models are needed to uncover plausible clinical potential of NHEJ targeting in these malignancies.

3.3. Colorectal cancer

Various findings that document genetic and signaling DNA-PK alterations in colorectal cancer (CRC) predict its role in the pathogenesis of this group of tumors. In an early 2001 study by Rigas et al., the expression levels of DNA-PK, Ku70 and Ku80 have been compared between normal and human colon cancer tissues (Rigas et al., 2001). DNA-PK levels were similar between both tissues but adenomas and carcinomas

showed a reduction of Ku70 and Ku80 expression compared to normal colon tissue, suggesting their implication in the development of colon cancer. Using a small cohort of patient samples, a 2004 study reported that DNA-PK activity as well as protein and mRNA levels of Ku70, Ku80 and DNA-PKcs were elevated in CRC tissues (Hosoi et al., 2004). As Ku80, Ku70 and DNA-PKcs have all consensus recognition elements for the SP1 transcription factor in their promoters, the authors postulated for a correlation between the elevated SP1 protein levels in tumor tissues and the DNA-PK status. Using a cohort of 37 CRC patients, all carriers of a KRAS codon 12 mutation, Ghezzi et al. reported in 2011 that Ku70 expression in poorly-differentiated tumors is significantly higher than in well- and moderately-differentiated colorectal tumors (Ghezzi et al., 2011). Additionally, downregulated Ku70 was linked with poor disease-free survival in CRC and its loss of expression was suggested to serve as a biomarker to predict poor prognosis (Lu et al., 2014). With respect to treatment outcome and by referring to data from a cohort of 96 patients with advanced rectal carcinoma, the expression pattern of Ku70 and Ku80 was shown as predictive for tumor responses to radiation therapy (Komuro et al., 2002). Two genetic DNA-PK SNPs have been reported in CRC: the Ku80 G1401T/SNP rs828907 was associated with the development of CRC (Yang et al., 2009a, 2009b) and the *PRKDC* intron 8 G6721T (SNP rs7003908) polymorphism with a significant increase risk in colon carcinogenesis (Sishc and Davis, 2017).

The potential role of DNA-PK in colon cancer has been also investigated in pre-clinical models of CRC. A small interfering RNA (siRNA) library screen with a subsequent validation through DNA-PKcs downregulation, identified *PRKDC* as an essential gene in CRC cells (S. Sun et al., 2016). The interference with DNA-PK signaling led to apoptosis, partially through AKT inhibition, and rendered CRC cells sensitive towards chemotherapeutic agents that interfere with DNA replication. Interestingly, a study from 2015 by Wilson et al. described a link between the AIM2 inflammasome, a member of innate immune sensors and a key player in host defence, and DNA-PK in colon carcinogenesis. Through the use of *Aim2*-deficient mice that exhibit high CRC burden, the study revealed that AIM2 interacts and limits the activation of DNA-PK, which promotes AKT phosphorylation. Consequently, the loss of AIM2 stimulated DNA-PK–mediated AKT activation, thereby enhancing tumor load (Wilson et al., 2015). In addition, the study by Nixon et al., which has been earlier introduced in the context of breast cancer, found also that CRC HCT-116 cells showed reduced expression of the new RAF1 splice variant, Raf1-tr, that associates with nuclear DNA-PK, and that its introduction into the cells sensitized them to bleomycin-induced apoptosis (Nixon et al., 2019).

The potential benefit of DNA-PK inhibitors has been studied in CRC for several years. For example, KU-0060648, a dual inhibitor of DNA-PK and PI3K, was shown to sensitize DNA-PK-deficient cells to etoposide and doxorubicin and to reduce cell proliferation *in vitro* and *in vivo* (Munck et al., 2012). More recently, the sensitivity to this compound was linked to the loss of MMR proteins, such as MSH3 and MLH1. Accordingly, these proteins have been suggested as biomarkers for the identification of patients who could benefit from this therapy (Hinrichsen et al., 2017). Moreover, the DNA-PK inhibitors NU7026 and IC486241 showed a synergistic effect with the irinotecan active metabolite, SN38 and oxaliplatin due to an increased DNA damage (Davidson et al., 2012a, 2012b). Within a study investigating properties of the highly selective and potent CHK1 inhibitor V158411, it has been observed that inhibition of CHK1 in HT-29 CRC adenocarcinoma cells activated DNA-PKcs as determined by increased its autophosporylation on Ser2056 (Massey et al., 2016). The study shows that high expression of DNA-PKcs, confers higher sensitivity to the CHK1 inhibitor V158411, suggesting thereby that high DNA-PKcs levels might help to stratify patients with tumors highly sensitive to CHK1 inhibitors.

Also, DNA-PK inhibitors have been combined with the oncolytic virus M1, reporting promising data as the inhibition of DNA-PK increases the DNA damage induced by the virus, resulting in increased cell death (Xiao et al., 2018). Interference with DNA-PKcs has also been suggested to sensitize CRC cells to mTOR inhibitors (Wu et al., 2015). Accordingly, DNA-PKcs Thr-2609 phosphorylation was shown to be critical for resistance of HT-29 CRC cells to the mTOR inhibitor WAY-600. The use of the DNA-PKcs inhibitors NU7026 and NU7441 significantly enhanced the WAY-600-induced cytotoxicity and pro-apoptotic effect. These observations denote DNA-PKcs signaling as a relevant resistance mechanism for mTOR targeting in CRC cells.

3.4. Gastric and esophageal cancer

As with tumors of other histotypes, promoter SNP variants of Ku70 and Ku80 were described also in gastric cancer. The Ku80 promoter G-1401 T (rs828907) polymorphism was investigated in the context with gastric cancer risk (J. Q. Li et al., 2011). Looking into a cohort of 241 patients, the findings suggest significant differences between gastric cancer and control groups in the distribution of frequencies ($P = .002$) in the G-1401 T polymorphism. Similarly, the Ku70 promoter T-991C (rs5751129) variant has been reported to link to gastric cancer predisposition (Yang et al., 2011).

Correlation between expression levels of DNA-PK and other NHEJ proteins with intervention responses has been studied also in esophageal and gastric cancers (Hori et al., 2017; Lee et al., 2005; Lee et al., 2007; Noguchi et al., 2002; Tonotsuka et al., 2006). Esophageal cancer patients with high DNA-PKcs-expressing tumors exhibited a greater therapeutic benefit following chemoradiation modalities compared with patients with lower levels of DNA-PKcs (Noguchi et al., 2002). Similarly, absence of DNA-PKcs expression has been associated with gastric cancer progression, presence of intra-tumoral neutrophils, poor survival (Lee et al., 2005; Lee et al., 2007) and lymph node metastasis (Lee et al., 2005). Notably, DNA-PK expression exhibited a high intratumoral heterogeneity in patient samples of esophageal tumors, suggesting that its activity or expression levels might not be good predictive biomarkers of radio- or chemotherapy sensitivity (Tonotsuka et al., 2006). Absence of DNA-PK expression was also determined in a subset of gastric cancers characterized by high microsatellite instability (MSI-H) and was shown to occur due a frameshift mutation of poly$(A)_{10}$ mononucleotide repeats (H. S. Lee et al., 2007). MSI-H gastric cancer patients were more likely to have lymph node metastasis and those carrying the poly$(A)_{10}$ mononucleotide mutation had an increased risk compared to non-carriers (H. S. Lee et al., 2007).

With respect to DNA-PK molecular targeting, plausible radiosensitizing effects of the DNA-PK inhibitor NU7026 were reported with the gastric cancer line N87 (Niazi et al., 2014). The combined NU7026/irradiation treatment showed a significant increase in the DNA damage marker histone γH2AX, in G2/M cell cycle arrest and in apoptosis compared to administration of irradiation alone. These results suggest that NU7026 enhances irradiation-mediated cytotoxic effect resulting in an increase of DNA DSBs that arrest the cell cycle at the G2/M checkpoint with consequent apoptosis (Niazi et al., 2014).

Recently, the role of the insulin-line growth factor binding protein 2 (IGFBP2) in esophageal adenocarcinoma (EAC) was determined (Zhou et al., 2019). The development of EAC has been linked to chronic gastro-esophageal reflux disease, which is characterized by the presence of acidic bile salts (ABS). In this context, endogenous IGFBP2 knockdown significantly increased DNA DSBs and apoptosis, while the overexpression of exogenous IGFBP2 had a protective effect against the DNA DSBs and apoptosis caused by ABS. To determine the DDR pathway involved in these findings, IGFBP2 was either knocked down or overexpressed, resulting in a decreased and increased, respectively, EGFR and DNA-PKcs phosphorylation. Moreover, co-immunoprecipitation analyses determined that IGFBP2, EGFR and DNA-PK co-localize in a protein complex upon ABS treatment and IGFBP2 knockdown decreased the interaction between EGFR and DNA-PK due to the unstable EGFR protein. Therefore, IGFBP2 expression may protect EAC cells against DNA DSB and apoptosis induced by ABS

through a mechanism that stabilizes EGFR to interact with DNA-PKcs for the signaling axis activation (Zhou et al., 2019).

3.5. Glioblastoma multiforme and neuroblastoma

The role of DNA-PK in the context of glioblastoma multiforme (GBM) and neuroblastoma has been investigated from different angles, as responses to DNA-damaging agents, association with clinical parameters and as a target for radiosensitization.

Early preclinical research in the field underlined the association of DNA-PK activation induced by staurosporine, ceramide and UV radiation with apoptosis onset in neuroblastoma cells (Chakravarthy et al., 1999) whereas studies by Virsik-Köpp et al. assessed the impact of DNA-PK deficiency on the formation of chromosome aberrations in irradiated glioblastoma (Virsik-Kopp et al., 2003). It has been demonstrated that radioresistant DNA-PK-proficient cell lines acquire less complex aberrations and acentric chromosome fragments compared to their DNA-PK-deficient counterparts and that DNA-PK inhibition by wortmannin increases the aberration yield in the DNA-PK-proficient model to the same qualitative and quantitative level as detected in the DNA-PK-deficient line (Virsik-Kopp et al., 2003). Analogous GBM cellular systems have been used to study the regulation of DNA-PK activity *via* the protein kinase CK2. Downregulation of distinct CK2 catalytic subunits resulted in cell death in DNA-PKcs-proficient M059K cells but not in the isogenic DNA-PKcs-deficient M059J line. Furthermore, neocarzinostatin treatment of M059K cells depleted of either of the CK2 catalytic subunits decreased Ser2056 DNA-PK autophosphorylation and inhibited DNA-PKcs-mediated repair of DSBs, events likely resulting from the disruption of a direct DNA-PK-CK2 interaction (Olsen et al., 2010).

Translational relevance of these and similar mechanistic findings is naturally dependent on the presence and activity of DNA-PK in patient tumor tissues. The 2011 data by Kase et al. indicated that DNA-PK levels correlate with post-irradiation survival of post-surgery glioblastoma patients ($n = 34$; 9.0 months for the group with low *vs.* 13.0 months for the group with high DNA-PK expression) (Kase et al., 2011). In the context of neuroblastoma, Saini et al. investigated mechanisms underlying de-differentiation of neuroblastoma cells within a proteomic study. The results validated by a tissue microarray of 30 neuroblastoma cases, and found that the expression of DNA-PKcs is increased in advanced stages (Saini et al., 2014). Correlation of enhanced *PRKDC* mRNA expression and poor survival in neuroblastoma patients reported by Dolman et al. provided a basis for their studies on the radiosensitization potential of the DNA-PK small molecule inhibitor NU7026 (Dolman et al., 2015). NU7026 formed a synergistic pro-apoptotic combination with a single irradiation dose of 0.63Gy that was increasing with time and reaching a maximum effect 96 h post irradiation (Dolman et al., 2015). Importantly, radiosensitization due to impaired NHEJ has been observed in several neuroblastoma cell lines but not in normal fibroblasts with low DNA-PK expression (Dolman et al., 2015).

Radiosensitization by the use of another DNA-PK small molecule inhibitor, VX984, has been recently investigated in *in vitro* and *in vivo* models of glioblastoma. VX984 increased radiosensitivity of adherent U251 and stem-like NSC11 cell lines in clonogenic assays and led to higher γH2AX levels and DSBs in neutral comet analyses (Timme et al., 2018). Moreover, VX984 in combination with IR increased overall survival in comparison to IR alone in mice with orthotopic GBM xenografts, supporting the evaluation of DNA-PK inhibitors in GBM in clinical trials (Timme et al., 2018).

3.6. Head and neck cancer (HNC)

Two SNP variants have been reported in the genes encoding Ku70 and Ku80 in head and neck cancers. The T-991C (SNP rs5751129) polymorphism located in the promoter region of the *XRCC6* gene that encodes for KU70 has been correlated with significantly lower mRNA and protein expression levels in tissues of nasopharyngeal carcinoma (NPC) patients (Huang et al., 2015). These data suggest that this Ku70 variant may play a role in the pathology of NPC and could serve as a factor for personalized medicine and therapy in this subset of HNC. An association between the Ku80 promoter G-1401 T (rs828907) with oral cancer risk was investigated in a cohort of 600 patients with oral cancer compared with matched healthy controls (Hsu et al., 2009). The findings published in 2009 suggested that Ku80 G-1401 T correlates with oral cancer susceptibility. Additionally, the expression levels of the DNA-PKcs protein have been investigated in 223 samples of NPC tissues (Yan et al., 2008). DNA-PKcs levels were found to be a prognostic predictor of NPC with poor survival outcomes and possibly may play a role in recurrent disease and metastasis.

As radiation therapy is a main therapeutic modality in HNC, it has been of interest and need to investigate markers associated with radiation response of these tumors. Considering their driving role in DDR, immunohistochemical expression of DNA-PKcs and Ku70/80 have been determined in purpose to assess their relation with patient and tumor characteristics and as plausible predictive markers (Bjork-Eriksson et al., 1999). However, no significant correlation has been found between DNA-PKcs and Ku70/Ku80 expression and the site of tumor, patient age and sex, histology, or radiation response (Bjork-Eriksson et al., 1999). In 2016, Lu et al. described a mechanism mediated through the Epstein-Barr virus-encoded protein LMP1, which attenuated DSBs repair in NPC through the inhibition of DNA-PK activity, a path, which also involved a disruption in the association between DNA-PK and the AMP-dependent protein kinase (AMPK) and associated reduced AMPK T172 phosphorylation (Lu et al., 2016). Eventually, those events result in LMP1-dependent glycolysis and resistance to radiation-mediated apoptosis.

Also in 2016, whole exome sequencing data analysis of head and neck squamous cell carcinoma (HNSCC) revealed aberrations in Fanconi anemia (FA) genes, leading to impaired DNA repair (Romick-Rosendale et al., 2016). Interestingly, further investigations have shown that FA pathway loss leads to cytoskeleton reorganization and invasive properties, a path mediated through RAC1 GTPase that can be reversed by DNA-PK inhibition (Romick-Rosendale et al., 2016).

A meta-analysis of HNSCC datasets revealed a possible role of the AAA + ATP-ase TRIP13/HPV16E1BP in treatment resistance of these tumors (Banerjee et al., 2014). This hypothesis has been further investigated by mass spectrometry, identifying DNA-PK as a TRIP13-binding partner and, interestingly, overexpression of TRIP13 in HNSCC has been associated with enhanced NHEJ activity and a consequent higher sensitivity to DNA-PK inhibitors (Banerjee et al., 2014). These observations point to TRIP13 overexpression as a therapeutic marker for the identification of DNA-PK inhibitors responders.

Establishment of a gene expression model analysis with the use of machine learning tools enabled to uncover association between DNA crosslink repair defects frequent in head and neck and aggressive tumors, their chemoradioresistant properties and a poor patient prognosis. The subsequent *in vitro* experiments employing RAD51 inhibition revealed induction of this aggressive phenotype while DNA-PK inhibition reduced it (Essers et al., 2019).

In a recent study comparing radiosensitization potency of PARP inhibitors (olaparib, veliparib) and DNA-PK inhibitors (KU57788, IC87361) in HNSCC cell lines, KU57788 and IC87361 have been more effective than olaparib and veliparib, especially under hypoxia (T. W. Lee et al., 2019). Additionally, the radiosensitizing effect of IC87361 was related and enhanced by the expression of the SLFN11/Schlafen-11 endoribonuclease that activates a cell death pathway in response to DNA replication fork damage (Murai et al., 2018; Zoppoli et al., 2012).

3.7. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a common human malignant disorder with a life expectancy of about 6 months as of the time of

diagnosis (Llovet and Bruix, 2008). A complex interaction between genetic and environmental risk factors determines a broad genotypic and phenotypic heterogeneity within human HCC (Donato et al., 2006; Dragani, 2010). Several modalities as partial hepatectomy, radiofrequency ablation, and liver transplantation are used as potential curative therapies (Sherman, 2011), however, only a small number of patients can undergo these treatments due to advanced disease stage at time of diagnosis (Calvisi et al., 2007; Llovet and Bruix, 2008; Sherman, 2011). Currently, there is an urgent need for understanding mechanisms underlying HCC progression that could provide novel opportunities for pharmacologic interventions.

Accumulating preclinical observations indicate an important role of DNA-PKcs in HCC establishment, progression and patients' prognosis. Although other types of liver cancers such as cholangiocarcinoma and biliary cystadenocarcinoma show deregulated DNA-PKcs expression, HCC is considered to have the highest expression of this critical NHEJ component (Evert et al., 2013). DNA-PKcs has also been suggested to be involved in HCC biology under low oxygen conditions as its levels have been shown to be upregulated under hypoxia in HepG2 hepatoma cells (Um et al., 2004a, 2004b). Moreover, DNA-PKcs may also interact with, and phosphorylate HIF-1α, suggesting a potential regulation of this hypoxia master transcription factor (Um, Kang, et al., 2004). Other lines of evidence suggest also a crosstalk between DNA-PKcs and MYC in HCC models. In that respect, Raymond et al. reported that DNA-PKcs inhibited the proteolysis of MYC by suppressing its proteasomal degradation in HepG2 cells (Raymond et al., 2015). The further relevance of DNA-PKcs in regulating MYC was also observed using the LO2 normal liver cells and demonstrating that overexpression of DNA-PKcs is followed by increased MYC levels and associated upregulation of AKT (Raymond et al., 2015).

Additionally to these findings, the heat shock transcription factor-1, identified as a potential novel target with increased mRNA and protein levels in human HCC, has been reported by Evert et al. to upregulate DNA-PKcs through the activation of the MAPK/JNK/AP-1 axis (Evert et al., 2013). Interestingly, and on the other hand, the regulatory DNA-PK subunit Ku80 has been described as a tumor suppressor in HCC as its overexpression suppresses cell proliferation *in vitro* and *in vivo* (Wei et al., 2012). Moreover, in these models, Ku80 overexpression arrests cell cycle at S-phase through the upregulation of p53 and p21, which can be reversed by p53 or p21 inhibition (Wei et al., 2012).

The association between DNA-PK levels/activation and different cellular endpoints such as proliferation or survival has been also assessed in HCC. Activated DNA-PKcs increases proliferation, genomic instability, microvessel density and has been associated with decreased apoptosis (Evert et al., 2013) and poor survival (Cornell et al., 2015). Moreover, high levels of DNA-PKcs have been identified in treatment-resistant HCC patients, whose disease progressed at a median of 4.5 months compared with 16.9 months (Cornell et al., 2015). This resistance was overcome by the use of the DNA-PKcs inhibitor NU7441, which suppresses HCC growth *in vitro* and *in vivo* in combination with irradiation or doxorubicin (Cornell et al., 2015).

Collectively, these results suggest that DNA-PKcs activity is potentially linked with pathways in the liver, which might have impact on HCC development, progression and treatment responses.

3.8. Lung cancer

An early Sirzén et al. pre-clinical report back in 1999 studied the correlation between DNA-PK levels in five lung carcinoma cell lines and response of the cells to radiation in terms of DSBs repair and radiosensitivity (Sirzen et al., 1999). The findings of this study suggested that the lowest DNA-PK levels/activity was found in the most radiosensitive cells, whilst the highest DNA-PK presence was associated with the lines displaying a radioresistant phenotype.

The expression of Ku80 in clinical lung adenocarcinoma specimens and its role in the regulation of cisplatin sensitivity has been investigated in a cohort of 106 patients with operable lung adenocarcinoma (Ma et al., 2012). The work found that Ku80 was markedly overexpressed in primary human lung adenocarcinoma and high Ku80 expression was associated with poor clinical outcomes and resistance to cisplatin-based chemotherapy. Complementary to the clinical data, siRNA-mediated knockdown of Ku80 enhanced the pro-apoptotic effects of chemotherapy of the cisplatin-resistant lung adenocarcinoma cells A549/DDP that overexpress Ku80.

In a 2001 study by Auckley and colleagues, DNA-PK activity has been correlated with carcinogenesis, progression as well as prediction of treatment response in lung cancer. In a case control study comparing stages I-IV newly diagnosed non-small cell lung cancer (NSCLC) patients and healthy individuals, the DNA-PK activity from peripheral mononuclear cells was significantly lower in patients as compared to healthy controls (Auckley et al., 2001). Importantly, this reflected the status of DNA-PK activity in bronchial epithelial cells that are precursor cells from which lung cancer may develop (Auckley et al., 2001).

Decrease in DNA-PK activity leading to genetic instability and lung carcinogenesis has been mechanistically associated with radiation-induced BCL-2 activation. BCL-2, a potent suppressor of apoptosis, was shown to directly interact with Ku70 and Ku80 *via* its BH1 and BH4 domains, leading to inhibition of DSBs repair *via* NHEJ (Q. Wang et al., 2008). BCL2 expression was shown to correlate with a reduced Ku activity in human lung cancer cells, advocating the possibility of BCL2 interference with the NHEJ pathway and the development of lung cancer.

Another reported mechanism for DNA-PK involvement in lung cancer is related to the suppressed activation of DNA-PKcs through the inhibitory effect of the Tudor domain-containing protein 9 (TDRD9). TDRD9 is a RNA helicase whose overexpression in lung adenocarcinoma has been related to hypomethylation of the CpG island within the 5′ regulatory region of the gene. (Guijo et al., 2018). *TDRD9* knockdown in lung cancer cells elicited the activation of DNA-PKcs.

Whereas low DNA-PK activity may predispose to the development of lung cancer, its higher activity on the other hand is likely used by cancer cells as a pro-survival path leading eventually to resistance towards DNA damage-based therapeutic modalities. By a quantitative real-time PCR analysis of 140 lung cancer patients tissues, it has been shown that DNA-PKcs is significantly higher in the tumor when compared with bordering normal tissue, a result linked with a more than twice increased risk of death (Xing et al., 2008). Interestingly, when examining mechanisms that account for DNA-damaging agents (DDAs) resistance of lung cancer stem-like cells, Lundholm et al. found that those cells display a lesser phosphorylation status of DDR effectors, as ATM, KAP1 and DNA-PK, indicating hence a reduced activity mode (Lundholm et al., 2013).

With respect to early anti-DNA-PKcs perturbations, NSCLC cells undergoing DNA-PKcs interference, either through down-regulation by antisense oligodeoxynucleotides or exposure to wortmannin, showed increased DNA DSBs and an enhanced radiation response (Sak et al., 2002). Similarly, a Ku70 antisense approach in a human squamous cell lung carcinoma cell line resulted in higher radiosensitivity and chemosensitivity to bleomycin and methyl methanesulfonate (Omori et al., 2002).

In a recent study using A549 human NSCLC cells, a treatment modality combining the novel DNA-PKcs small molecule inhibitor M3814 along with carbon ion irradiation elicited high efficacy in eliminating radioresistant hypoxic tumor cells (Klein et al., 2017).

As EGFR mutations and receptor amplification are relevant to the lung cancer genomic landscape, the EGFR-DNA-PK axis has been studied in the context of responses to DNA damage-based treatment. Accordingly, Javvadi et al. reported a modulatory role for EGFR signaling in DNA-PK-mediated DSB repair and radiation resistance of NSCLC. The model for the EGFR-DNA-PK signaling interphase suggests that initial IR-induced ATM-dependent DNA-PKcs phosphorylation at Thr2609 is required for the interaction between nuclear EGFR and DNA-PKcs. Moreover, the authors also found that EGFR regulates DNA-PKcs

function through stabilization of the Thr2609 phosphorylation. Activating EGFR mutations as L858R or the ΔE746-E750 in-frame deletion prevent EGFR-DNA-PKcs interaction and compromise the stability of Thr2609 phosphorylation that negatively affects eventual DSBs repair (Javvadi et al., 2012). Similar NSCLC data showing the failure of EGFR variants EGFR L858R and ΔE746-E750 to translocate to the nucleus, to associate with DNA-PKcs and to elicit an EGFR-linked radioprotection were reported also in an earlier work (Das et al., 2007). Taken together, these observations suggest a careful stratification for the application of EGFR-based radiosensitization protocols for NSCLC patients, which should take into consideration the tumor *EGFR* status. Similar to other tumors, activation of DNA-PK *via* AKT was also reported in the NSCLC cell lines A549 and H460 (Toulany et al., 2008). In these models, AKT signaling perturbation by AKT-targeting siRNA leads to failure of DSBs repair (Toulany et al., 2008).

In a recent 2019 manuscript by Fok et al., another novel selective DNA-PK inhibitor, AZD7648, demonstrated potent activity in both *in vitro* and *in vivo* models consisting of the A549 and H1299 lung adenocarcinoma cell lines, in particular in the context of radiosensitizing features (Fok et al., 2019). The *in vitro* data confirmed that AZD7648 leads to the persistence of DNA damage following IR, resulting in G2/M DNA damage checkpoint activation, genome instability and reduced cellular survival. When tested *in vivo*, AZD7648 that was administered orally enhanced the response to fractionated IR in mice with A549 or H1299 NSCLC xenografts. While tumors were insensitive to single-agent AZD7648 treatment, IR treatment alone induced tumor growth inhibition by 50%, but a combination of AZD7648 with IR achieved 90% of growth inhibition. Parallel to these biologic consequences in these NSCLC models, the IR-induced phosphorylations of DNA-PKcs at Ser2056, γH2AX and RPA32 at Ser4/Ser8 were efficiently blocked by AZD7648, indicating therefore the stabilization of the DNA damage through the DNA-PKcs perturbation.

In summary and taking into account the different mechanistic aspects of DNA-PK activation in lung cancer, its therapeutic, prognostic and predictive role need yet to be fully understood and assessed in terms of precision oncology concepts for achieving a maximal impact in current lung cancer management.

3.9. Lymphoma

Lymphoid tumors were among the first in which a role for the DNA-PK system has been elucidated, primarily through the use of preclinical models. An early biochemical characterization of DNA-PKcs with respect to its autophosphorylation has been done in context of lymphoma cells nearly 30 years ago by Iijima et al. Using Raji Burkitt's lymphoma cells the work showed that DNA-PK autophosphorylation induces serine phosphorylation of MYC, and that this activity requires the presence of double-stranded DNA (Iijima et al., 1992).

Homozygous Ku70 knockout mice develop T cell lymphomas, whereas heterozygous littermates remain tumor-free. This observation supports the potential function of the DNA-PK regulatory subunit Ku70 as a tumor suppressor gene for T cell lymphoma development (G. C. Li et al., 1998). Further early studies employing knockout mice of the catalytic subunit DNA-PKcs were instrumental in sheding light over DNA-PKcs role in the immune system development. DNA-PKcs-null mice show abnormalities in V(*D*)J recombination, increased radiosensitivity and SCID-like phenotype but no increase in development of thymic lymphoma has been observed (Kurimasa et al., 1999). Instead, hyperplastic polyps and aberrant crypt foci have been found in the intestines of DNA-PKcs-null mice, suggesting similarly function of a tumor suppressor gene (Kurimasa et al., 1999). Along the same line, Espejel et al. have reported increased incidence of T cell lymphoma, shortened telomers and earlier aging of DNA-PKcs-deficient mice on a longer follow-up analysis (Espejel et al., 2004).

As in the context of tumors of other origins, numerous studies on lymphoma have investigated the role of DNA-PK in responses to DDAs. As anticipated, allelic variations in the *PRKDC* gene coding for DNA-PKcs resulted in differences in radiation sensitivity and radiation-induced lymphomagenesis (Mori et al., 2001). Interestingly, a correlation between BCR-ABL, the fusion that drives chronic myelogenous leukemia, and DNA-PKcs has been reported by Deutsch et al. in 2001. Ectopic expression of BCR-ABL in haematopoietic cells was accompanied with strong decrease of DNA-PKcs levels. Accordingly, proteasome-mediated DNA-PK degradation has been observed in stable and inducible BCR-ABL-expressing cell lines, leading to genetic aberrations and sensitivity to irradiation (Deutsch et al., 2001). A potential vicious circle of defective DNA repair together with antiapoptotic activity was shown to contribute to a blast crisis that could be reversed by proteasome inhibitors through the prevention of DNA-PKcs degradation (Deutsch et al., 2001).

Untreated lymphoma patient samples have been investigated in the context of DNA-PKcs, Ku80, and Ku70 expression. According to data reported by Holgersson et al., higher DNA-PKcs and Ku80 expression is present in more aggressive tumors such as acute myeloid leukemia, high-grade lymphoma, multiple myeloma, and is contrasting with their lower expression in chronic myeloid leukemia and low-grade lymphoma (Holgersson et al., 2004).

NK314, a dual inhibitor of topoisomerase IIα and DNA-PK, was reported to overcome resistance to topoisomerase inhibitors in DNA-PK-expressing M059K T-cell leukemia-lymphoma cell line, having a similar effect as a combination of etoposide and NU7026, specific topoisomerase and DNA-PK inhibitors, respectively (Hisatomi et al., 2011). DNA-PK has been shown to be involved in olaparib-induced cytotoxicity in ATM- and p53-mutated mantle cell lymphoma cells, acting as a stabilizing agent for mutated p53 (Williamson et al., 2012). Those data also correspond with experimental findings using mouse models lacking PARP1 and DNA-PK, showing their protective role in lymphoma formation when p53 is mutated (Rybanska et al., 2013). Recently, early clinical trials on dual TORK/DNA-PK inhibition (NCT01353625) have shown promise for relapsed/refractory chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma with ATM deletions/mutations (Thijssen et al., 2016).

3.10. Melanoma

As etiology of melanoma is often linked to environmental factors such as sun exposure, alterations in genes that repair exogenous DNA damage, including the NHEJ pathway, might increase the disease risk. Indeed, evaluation of DNA repair genes variants in 586 individuals from 53 melanoma high-risk families has revealed four different *PRKDC* polymorphisms (the most significant one being rs8178158) as well as SNPs in the POLN gene as being significantly associated with this pathology (Liang et al., 2012). When testing if levels of distinct key DDR and replication proteins are prognostic biomarkers in melanoma, Song et al. found that *XRCC5* (Ku80) overexpression relates to significantly worse survival (Song et al., 2013). At that time it has been already shown that metastatic melanoma cells express high levels of proteins with potential antiapoptotic effects including DNA-PK and EGFR and that the mutually regulated activities of these two kinases implicate their role in the malignant phenotype and treatment resistance (Um et al., 2004a, 2004b).

Important mechanistic insights into metastasis regulation *via* DNA-PKcs were provided by a 2015 preclinical study by Kotula et al. who reported that increased DNA-PKcs activation stimulates angiogenesis, melanoma cells migration ability and metastasis that can be reversed by DNA-PK inhibition (Kotula et al., 2015). Interestingly, DNA-PKcs pro-metastatic activity in melanoma is executed by regulation of secretion rather than gene expression, of more than 80 distinct proteins with a consequent possible modification of the tumor microenvironment (Kotula et al., 2015). These findings are further supported by experimental indications that the modulation of DNA-PK function influences immunobiology of both tumor as well as T cells. In a high-throughput

flow cytometry-based screening, the DNA-PK inhibitor NU7441 was shown to favorably alter a variety of immunomodulatory proteins including increase in HLA-I expression and PD-L1 reduction in a heterogeneous panel of melanomas (Tsai et al., 2017). Following NU7441 treatment, T cells expressed increased levels of activation markers, costimulatory molecules, cytokines and decreased expression of the coinhibitory receptors., thus conditioning their better response to T-cell based immunotherapy (Tsai et al., 2017). Such data are highly promising in light of the fact that immunotherapies, which have revolutionized melanoma treatment, lead as single-agent treatments to complete regressions only in 5–20% of patients (Ascierto et al., 2019; Robert et al., 2015).

Besides immunotherapies, targeting of the V600E-mutated BRAF found in *ca.* 60% of melanoma cases has, often in combination with MEK inhibitors, markedly improved patients' survival. However, due to a rapid acquisition of therapy resistance, efficacies of new treatment strategies and combinations, including DNA-PK targeting, are constantly being assessed (N. Li et al., 2017; Margue et al., 2019; Shih et al., 2016).

Although equally of melanocyte origin, uveal melanoma (UM), arising intraocularly, is a considerably different malignancy in terms of genetic background as compared to the cutaneous form (van der Kooij et al., 2019). In a recent study by Doherty et al., low sister-chromatide exchange rate observed in UM cell lines prompted the investigators to assess mRNA expression of NHEJ-related genes in the UM TCGA dataset. Apart of an increased expression of *PRKDC*, *XRCC5* and *XRCC6* in UM, elevated NHEJ activity and DNA-PKcs activation has been detected (Doherty et al., 2019). UM models have shown an increased sensitivity and cell death towards DNA-PK-mediated inhibition of NHEJ by NU7026 or NU7441 administered as single agents as well as in combination with IR or inter-strand cross-linking agents as compared to other cancer types including cutaneous melanoma (Doherty et al., 2019). In another recent study by Dogrusöz et al., the authors evaluated the expression of DNA repair-related genes in the Leiden cohort of 64 UMs and identified *PRKDC* as a gene, which was highly expressed in cases with unfavorable prognosis (Dogrusoz et al., 2019). DNA-PK inhibition by NU7026 led to downregulation of the EMT-related transcription factor SNAIL1 and to attenuation of proliferation in the primary as well as in metastatic UM cell lines. These data strongly imply further investigations on DNA-PKcs as a potential therapeutic target in UM.

3.11. Ovarian cancer

Based on Genomic Data Commons (GDC) data, ovarian cancer ranks as a cancer type with the second most cases (27%) harboring *PRKDC* CNV events (Fig. 1). Elevated expression of DNA-PKcs in ovarian serous adenocarcinoma tissues associates with advanced disease, less favorable prognosis (Shin et al., 2016) and poor ovarian cancer-specific survival (Abdel-Fatah et al., 2014a, 2014b).

Certain insights into DNA-PK role in ovarian cancer therapeutic responses were provided by data of the Multicentre Italian Trial in Ovarian Cancer-2 (MITO-2), which enrolled 820 advanced ovarian cancer patients assigned to receive carboplatin/paclitaxel or carboplatin/PLD (Perrone et al., 2016). Although the two arms did not show difference in progression-free and overall survival and no biomarker had significant prognostic value as a result of tissue microarray analysis including 229 patients, high levels of DNA-PK and phosphorylated acetyl-coenzyme A carboxylase correlated with negative prognosis in the carboplatin/paclitaxel arm (Perrone et al., 2016).

As high-grade serous ovarian cancer is usually p53-mutated, genomically unstable and develops treatment resistance, some aspects of preclinical research on DNA-PK in ovarian cancer have been focusing on its role in tumor cell fitness and in therapeutic resistances. In this respect, Langland et al. that have profiled radiation response of a panel of 16 ovarian cancer cell lines reported that while their radioresistance is primarily determined by mutated p53, the DNA-PK status of the cells defined through gene copy number and expression levels kinase activity for DNA-PK, have a lesser impact on their radiosensitivity (Langland et al., 2010). On the other hand, early studies have already supported an involvement of DNA-PK in ovarian cancer resistance to platinum-based therapies. Cisplatin-induced decrease in DNA-PKcs kinase activity, which was significantly higher in cisplatin-resistant cell lines, reflected the extent of proteolysis of the catalytic subunit of DNA-PK (Henkels and Turchi, 1997). At the same time, impaired DNA-binding ability of the DNA-PK Ku subunits mirroring their decreased expression levels, was observed in apoptotic ovarian carcinoma cells following cisplatin treatment (Henkels and Turchi, 1997). In a later study by Townsend et al., DNA-PK overexpression in human ovarian carcinoma cells was linked with cisplatin resistance (Townsend et al., 2002). The work showed that cisplatin resistance could be overpowered by TLK286, a Glutathione S-Transferase π-activated prodrug of an alkylating agent. TLK286-induced destabilization of DNA-PKcs and the Ku heterodimer implicated DNA-PK inhibition as a potential mechanism of TLK286 cytotoxicity (Townsend et al., 2002). Later, Stronach et al. reported that DNA-PK phosphorylates Ser473 of AKT in response to cisplatin treatment in ovarian cancer resistant cell lines and suggested that their resensitization *via* DNA-PK inhibition is an alternative strategy to AKT inhibition that leads to toxicities independent of the DNA repair pathways (Stronach et al., 2011).

A potential role of DNA-PK in paclitaxel chemoresistance has been documented following ovarian cancer cell resensitization through the overexpression of the NOTCH3- and DNA-PK-targeting miR-136 (Jeong et al., 2017). Furthermore, McCormick et al. have recently studied association between defects in NHEJ and resistance to PARP inhibition in 6 cancer cell lines and 47 primary cultures of ovarian cancer (McCormick et al., 2017). Overall, 40% of the cultures had defective NHEJ and did not respond to treatment with the PARP inhibitor rucaparib whereas cells with defective HR repair and competent NHEJ were rucaparib-sensitive (McCormick et al., 2017). In these lines, the DNA-PK inhibitor NU7441 caused a rucaparib resistance in all sensitive cultures independently of their HR functionality, outlining the importance of NHEJ status for PARP inhibition efficacy in management of ovarian cancer.

Although no general conclusions on the utility of DNA-PK inhibition in the context of cytotoxic treatments of ovarian cancer can be drawn from these studies, the rather clear association between DNA-PK overexpression and worse patients prognosis and therapeutic responses warrants further exploring of DNA-PK targeting in particular settings. In 2019, Fok et al. reported on the effectiveness of the combination of the novel selective DNA-PK inhibitor AZD7648 and doxorubicin in ovarian cancer cells (Fok et al., 2019). The treatment decreased DNA-PKcs Ser2056, H2AX Ser139 (γH2AX) and the replication protein A subunit 32 (RPA32) Ser4/Ser8 phosporylations at early time-points and later resulted in increased levels of γH2AX and cleaved PARP1 as well as in the reduction in cell viability as compared to doxorubicin alone. Wise et al. have reported similar findings by using the DNA-PK inhibitor M3814 combined with PLD in ovarian cancer xenograft models (Wise et al., 2019) and this treatment combination is currently under investigation in the clinical trial NCT04092270.

3.12. Prostate cancer

Prostate cancer is one of the tumor entities in which the role of the DNA-PK complex in the pathogenesis, progression and determination of factors affecting responses to therapeutic modalities has been extensively studied. This can be at least partly attributed to the comprehensive findings evidencing the intricate crosstalk between the DNA-PK complex and the androgen receptor (AR) system, a signaling system that has a major driving role in prostate tumorigenesis (Debes and Tindall, 2002).

One of the first observations linking DDR alterations in a prostate cancer model with responses to DDAs is a 2004 report by Li et al. The

study showed that DU145 prostate carcinoma cells that carry a homozygous mutated hMLH1 mismatch repair gene with consequent MSI, affecting the genes for DNA-PKcs and two MRN complex effectors, RAD50 and MRE1, exhibit hypersensitivity to the radiomimetic bleomycin (Li et al., 2004).

As aforementioned, the understanding of the relevance of DNA-PK to prostate cancer biology has been significantly enhanced with the identification that DNA-PK interacts with the AR. The original 2005 Mayeur et al. study used tandem mass spectroscopy to identify AR to directly interact, *via* its ligand-binding domain with both Ku70 and Ku80, and indirectly with DNA-PKcs (Mayeur et al., 2005). Importantly, the work provided the critical evidence that both the Ku regulatory subunits and DNA-PKcs act as AR co-activators by enhancing its activity are involved in the AR transcriptional process. Further support for the role of DNA-PK in AR-related transcription was provided by the binding of its Ku regulatory subunits to the AR-responsive prostate specific antigen (PSA) promoter (Mayeur et al., 2005).

The liaison between AR and DNA-PK could be indirectly also implied from the effect of doxazosin, an alpha-1-adrenergic inhibitor that induces apoptosis of LNCaP androgen-dependent prostate cancer cells (Arencibia et al., 2005). Microarray analysis for gene expression changes in doxazosin-treated cell lines revealed downregulation of *XRCC5* and *PRKDC* (Arencibia et al., 2005), suggesting a potential relevance for the depletion of Ku80 and DNA-PKcs in survival of an androgen-dependent prostate cellular model.

A further insight as to the functional role of DNA-PK and AR interaction has been demonstrated by a digitonin-based permeabilized cell assay of the LNCaP prostate cancer cell line (Shank et al., 2008). DNA-PK activity was shown to impact AR sub-cellular localization *in vitro* through the use of a panel of activating ds-oligonucleotides. Subsequently, the AR nuclear export into the cytoplasm in this system could be blocked with the DNA-PKcs inhibitor NU7026 (Shank et al., 2008).

A major significant contribution to the understanding of the mechanisms and consequences of the AR-DNA-PK signaling intersection, particularly in response to treatment modalities that act by eliciting genotoxic damage, was provided by the Goodwin et al. study from 2013. This work demonstrated that androgen deprivation impedes the ability of the AR to promote DNA damage repair. Essentially, DNA-PKcs has been identified as a key target of AR after DNA damage and the AR-DNA-PKcs circuit as a major effector of DNA repair and therapeutic resistance in prostate cancer (Goodwin et al., 2013). Active AR was also shown to stimulate DNA-PKcs activity, as determined by increased DNA-PKcs phosphorylation on Ser2056 and DNA-PKcs-specific function. Reciprocally, DNA-PK was shown to potentiate AR function along with DNA repair and transcriptional regulation of AR (Goodwin et al., 2013).

An additional important observation supporting the interplay between the AR and DNA-PK systems was provided by Al-Ubaidi et al. in 2013. The study that investigated biopsies from prostate cancer patients who underwent castration therapy looked into Ku70 status in the analyzed tissues. The authors reported that Ku70 binds directly to the AR and that following castration therapy a reduction in Ku70 levels has been observed in 12 out of 14 patients (Al-Ubaidi et al., 2013).

Another comprehensive 2015 study provided evidence supporting DNA-PKcs as a direct master regulator of pro-metastasis programs in prostate cancer (Goodwin et al., 2015). The work showed a direct activity of DNA-PKcs in the modulation of prostate cancer metastasis networks with consequent promotion of tumor migration and invasion. DNA-PKcs has been found to be upregulated in clinical samples of advanced tumors and to be an independent marker for recurrent disease, metastasis and poor disease prognosis. As a major proof of principle, DNA-PKcs targeting with the specific inhibitor NU7441 was efficient to reduce tumor dissemination and metastasis formation in an *in vivo* model in which prostate tumor cells were injected systemically through the tail vain. Further supportive evidence in this direction was provided using patient tumor tissues that were used *ex vivo* as explants and treated with DNA-PKcs inhibitor. NU7441 in this system effectively reduced the expression of metastatic effectors as PREX1, ROCK2, ITGB4, and VAV3 (Goodwin et al., 2015).

In an attempt to get closer insights into networks which account for prostate cancer progression, a 2019 study by Kothari et al. investigated the overall kinome gene expression from a cohort of 545 patients and matched the resulting transcriptional profiles with patients long term clinical follow up data (Kothari et al., 2019). The data confirmed DNA-PK expression to be highly correlated with metastatic prostate cancer progression in high-risk cancer. Enrichment analysis identified the WNT pathway as a major DNA-PK interactor with consequent activation of the WNT-associated LEF1 transcription factor. Interestingly, DNA-PKcs targeting resulted in growth suppression of both androgen-dependent and -independent tumor cells. These novel data further substantiate DNA-PK as a master regulator of prostate cancer progression and suggest WNT targeting as an additional potential intervention modality.

The identification of a positive feedback signaling circuit encompassing AR-mediated expression and activation of DNA-PKcs and a reciprocal DNA-PKcs activation of the AR suggests that targeting the AR along with DNA-PKcs inhibition and a genotoxic perturbation might emerge as an effective therapeutic modality in prostate cancer. A related 2017 study that investigated the relevance of AR splice variants (ARVs) to prostate cancer treatment resistance found that ARVs induced through androgen deprivation promote post-irradiation prostate tumor cells survival through direct binding to DNA-PKcs (Yin et al., 2017). DNA-PKcs targeting by the specific inhibitor NU7441 significantly increased cellular DSBs as indicated through γH2AX levels and was accompanied with enhanced prostate cancer cell death after irradiation.

In a recent 2019 study, Chatterjee et al. aimed at investigating the mechanisms involved in the paradoxical phenomenon of growth inhibition of prostate cancer by supraphysiologic (SPA) androgens levels (Chatterjee et al., 2019). Using various prostate cancer models, the authors' findings support a critical role for DNA-PKcs in modulating tumor cytotoxicity through a SPA-induced, AR-mediated DNA damage mechanism. Mechanistically, the study showed that SPA reduces Thr2609 phosphorylation, a step, which is required for DNA-PKcs dissociation from the chromatin, thereby hindering the completion of DNA repair *via* NHEJ, resulting in elevated and persistent levels of cellular DNA damage. This study provides data to support a clinical evaluation of SPA in combination with DNA-PKcs inhibitors and in patients with AR amplification.

Another mechanism through which DNA-PK may affect cell survival in prostate cancer involves the phosphorylation of IGF-binding protein 3 (IGFBP-3) (Cobb et al., 2006). In that respect, Cobb et al. have identified and characterized IGFBP-3 phosphorylation on Ser156 by DNA-PK to be a critical step in the growth-inhibitory and apoptosis-promoting activities of IGFBP-3. DNA-PK-mediated phosphorylation of IGFBP-3, enhanced its nuclear accumulation and was found as a critical event for its interaction with its nuclear binding partner RXRα to exert pro-apoptosis activity.

Notably, DNA-PKcs has been implicated, similarly to PARP1, in mediating the oncogenic effects of the ETS transcription factor fusion product, *TMPRSS2:ERG,* which is predominant in prostate cancer (Brenner et al., 2011). Importantly, the ERG-DNA-PKcs association has been found in human prostate cancer tissues only in ERG gene fusion-positive cases. Site-directed mutagenesis revealed that Tyr373 of ETS is critically involved in the direct binding to DNA-PKcs. As to the functional significance of the physical association between DNA-PKcs and ETS gene fusion products, the study found it is entailed for ETS-mediated downstream transcription as well as cell invasion and metastasis.

In order to get into further signaling circuits related to DNA-PK signaling, Dylgjeri et al. used DNA-PK inhibition in a series of various prostate cancer *in vitro* models as well as xenografts and patient-derived explants to reveal novel DNA-PK networks *via* transcriptomics (Dylgjeri et al., 2019). Data analysis indicated the modulation of pathways known to be regulated by DNA-PK, including androgen responses,

estrogen signaling, cell cycle, and proliferation pathways. Newly identified DNA-PK-mediated pathways included EMT regulation, immune response, and metabolic processes. The study also suggests the investigation of a combinatorial strategy targeting DNA-PK/TOR kinase (TORK) and AR, which is currently being evaluated in the clinical setting in castration-resistant prostate cancer (NCT02833883).

From a prognostic perspective, expression of DNA-PKcs in prostate cancer predicts recurrence after radiation therapy (RT) independently of Gleason score as evidenced from TMA analysis of samples from 179 patients, where biochemical recurrence has been associated with positive DNA-PKcs nuclear staining (Bouchaert et al., 2012). Similar tendency of correlation has been shown with biochemical recurrence after permanent Iodine 125 interstitial brachytherapy in an analysis of 983 patient samples (Molina et al., 2016).

With respect to SNP variants, an intron 8-associated *PRKDC/XRCC7* polymorphism G6721T/SNP rs7003908 has been identified to correlate with a significantly increased risk of prostate carcinogenesis (Mandal et al., 2010). The functional relevance of this polymorphism remains still undefined, however, it has been predicted to regulate splicing and lead to mRNA instability or form a haplotype with other genetic changes in other genes through a linkage disequilibrium mechanism (L. E. Wang et al., 2004).

DNA-PK activity has also been used as a readout for local tumor control by RT in prostate cancer patients. DNA-PK activity in peripheral blood lymphocytes has been positively correlated in a cohort of 69 patients that were treated with 3D conformal radiotherapy and intensity-modulated radiation therapy, in the context of treatment results from the local tumor control perspective and normal tissue toxicity (Someya et al., 2017). Those data suggest the possibility for assessing RT efficiency through a DNA-PK function *via* a simple blood test.

Altogether, these findings implicate DNA-PK to have crucial pleiotropic and highly intricated roles in prostate cancer onset, progression and treatment responses. The significant findings that demonstrate a tight signaling interphase between DNA-PK and the AR system suggest treatment modalities that consist of targeting both those driving molecular components along with DDAs as a potential powerful strategy to be instigated in prostate cancer. Implementation of precision oncology-based stratifications would be necessary in order to ensure selection of patients for specific treatment combination modalities.

4. DNA-PK as a therapeutic target

Abnormal expression and deregulation of DNA-PK function in various human malignancies and its interference with the activity of therapeutic DDAs stimulated development of multiple DNA-PK inhibitory strategies. Due to significant structural homology of DNA-PK and PI3K, early attempts to interfere with DNA-PK activity were largely relying on small molecule inhibitors directed against PI3K and their derivatives. In addition, development of novel anti-DNA-PK approaches was utilizing a homology model of its ATP-binding site, DNA-PKcs-targeting microRNAs (Piotto et al., 2018; Yan et al., 2010) or antibodies and inhibitors specific to the Ku heterodimer such as ScFv 18–2 or compound L (Weterings et al., 2016; Xiong et al., 2012). In this chapter we will predominantly focus on the development of pharmacological approaches interfering with the catalytic activity of DNA-PKcs as these compounds are currently the most readily applicable in clinics. Whereas ongoing clinical trials (Table 2) study the effects of a handful of these inhibitors mostly in combination with chemo- or radiotherapy in distinct cancer types, the roles of DNA-PK in tumor-associated processes outside of DNA repair and in cancers with particular genetic deficiencies still need to be thoroughly exploited.

4.1. Identification and evaluation of preclinical DNA-PK inhibitors

Prime interest to identify small molecule catalytic DNA-PK inhibitors was motivated by the role of this enzyme in cellular DNA repair and such therapeutic approaches were thus assumed to act as potent radio- or chemosensitizers. Over the past two decades, numerous DNA-PK inhibitors greatly differing in their potency, selectivity and inhibition reversibility have been described. This includes the very early non-specific compounds such as caffeine (Sarkaria et al., 1999) or wortmannin (Izzard et al., 1999; Sarkaria et al., 1998) as well as highly potent selective inhibitors that are evaluated in current clinical trials (Fig. 4).

4.1.1. Early DNA-PK inhibitors

Sarkaria et al. reported already in 1999 that the radiosensitizing agent caffeine inhibits *in vitro* kinase activities of the two DDR master kinases ATM and ATR (Sarkaria et al., 1999). Later on it has been found that caffeine also inhibits immunoprecipitated and purified DNA-PK as well as DNA-PK in cell extracts (Block et al., 2004). The fungus-derived steroid metabolite wortmannin, which non-specifically inhibits PI3K family kinases, was also identified as a relatively potent inhibitor of DNA-PK and ATM activities (Sarkaria et al., 1998). Wortmannin forms covalent adducts with Lys3751 of DNA-PKcs's kinase domain and inhibits its activity *via* a non-competitive mechanism (Izzard et al., 1999). In 2003, Durant and Karran showed that a naturally occurring food component vanillin and its derivatives block DNA end joining by direct inhibition of DNA-PK activity and significantly potentiate cisplatin-mediated cytotoxicity (Durant and Karran, 2003). Although often used as tool compounds, the poor selectivity, water solubility or relative structural complexity of these early anti-DNA-PK molecules restricted their use to preclinical studies.

4.1.2. LY294002 as a lead compound

Due to high homology of their kinase domains, the search for DNA-PK inhibitory strategies was to a high extent paralleling development of specific PI3K inhibitors. At the onset of these attempts was quercetin, a naturally-occuring flavonoid that acts as an ATP-competitive antagonist for the kinase domain of PI3K and other kinases. In 1994, following a screen of quercetin-derived molecules, the chrome-4-one compound LY294002 (2-(4-morpholinyl)-8-phenylchromone) has been reported as a specific inhibitor of PI3K that equally potently inhibits also DNA-PK ($IC_{50} = 1.5$ μM) and mTOR (Vlahos et al., 1994) (Griffin et al., 2005).

Later on, LY294002 became a lead compound to develop more specific and more potent ATP-competitive DNA-PKcs inhibitors such as NU7441 (IC_{50} *vs.* DNA-PK = 13 nM), NU7427 (40 nM), NU7026 (0.23 μM) or NU7163 (0.19 μM) (Griffin et al., 2005; Hardcastle et al., 2005; Hollick et al., 2003; Leahy et al., 2004) (Fig. 4B). Preclinical evaluations of these novel potent kinase inhibitors revealed their radio- and chemosensitization potential when combined with external DNA damage. NU7427 sensitized HeLa cells to IR *in vitro* and potentiated cytotoxicity of the topoisomerase II inhibitor etoposide (Hardcastle et al., 2005) whereas NU7026 was shown to increase cytotoxicity of various topoisomerase II poisons in leukemia cells (Willmore et al., 2004). The more potent NU7441 increased the cytotoxicity of IR and etoposide in DNA-PKcs-proficient but not in DNA-PKcs-deficient human colon cancer cells regardless of their p53 status, substantially retarded the repair of IR- and etoposide-induced DSBs and appreciably increased G2/M cell cycle phase accumulation induced by IR, etoposide, and doxorubicin (Zhao et al., 2006). *In vivo*, NU7441 increased etoposide-induced tumor growth delay without exacerbating etoposide toxicity (Zhao et al., 2006). Subsequently, numerous independent studies utilizing NU7441 as a DNA-PK inhibitor in preclinical models reported similar observations in context of various tumor types such as breast cancer, NSCLC, liver cancer or leukemia (Alikarami et al., 2017; Ciszewski et al., 2014; Cowell et al., 2005; Tichy et al., 2014; Yanai et al., 2017; Yang et al., 2016).

Unfortunately, clinical testing and implementation of these compounds could not be pursued due to their suboptimal pharmacological profiles. For example, based on pharmacokinetic simulations, Nutley et al. predicted that NU7026 would have to be administered four

Table 2
Specific DNA-PK inhibitors in clinical trials (data from www.clinicaltrials.gov (last accessed on 30th March 2020)).

Trial	Drug	Target	Combinations	Cancer type	Phase
NCT01353625	CC-115	DNA-PK/mTOR	Monotherapy	GBM, HNSCC, prostate, Ewing's osteosarcoma, CLL, neoplasm metastasis	Phase I
NCT02833883	CC-115	DNA-PK/mTOR	Enzalutamide	Prostate	Phase I
NCT02977780	CC-115	DNA-PK/mTOR	Radiotherapy	GBM	Phase II
NCT02316197	M3814	DNA-PK	Monotherapy	Advanced solid tumors, CLL	Phase I
NCT02516813	M3814	DNA-PK	Radiotherapy, cisplatin	Advanced solid tumors	Phase I
NCT03724890	M3814	DNA-PK	Avelumab, radiotherapy	Advanced solid tumors	Phase I
NCT03770689	M3814	DNA-PK	Capecitabine, radiotherapy	Rectal	Phase I/II
NCT03983824	M3814	DNA-PK	Mitoxantrone, etoposide, cytarabine	AML	Phase I
NCT04172532	M3814	DNA-PK	Radiotherapy	Pancreatic	Phase I/II
NCT04092270	M3814	DNA-PK	PLD	Ovarian	Phase I
NCT04071236	M3814	DNA-PK	Radium-223 dichloride, avelumab	Prostate	Phase I/II
NCT04068194	M3814	DNA-PK	Avelumab, radiotherapy	Advanced solid tumors, hepatobiliary malignancies	Phase I/II
NCT04266912	M3814	DNA-PK	Avelumab	Advanced solid tumors with DDR genes aberrations	Phase I/II
NCT03907969	AZD7648	DNA-PK	Monotherapy, PLD, olaparib	Advanced malignancies	PhaseI/IIa

times per day at 100 mg/kg i.p. in order to obtain the drug exposure required for radiosensitisation (Nutley et al., 2005). An attempt to optimize pharmacokinetics of these compounds led to synthesis of very potent NU7441 analogs (IC_{50} *vs.* DNA-PK = 8 nM) (Cano et al., 2010), suggesting that such modifications could lead to the desired outcome.

Homology model of the ATP-binding site of DNA-PK based on PI-3 Kγ guided subsequent design of small molecule inhibitors with improved characteristics such as KU-0060648 (Cano et al., 2013; Clapham et al., 2012). Although KU-0060648 displayed very promising biological activity (IC_{50} *vs.* DNA-PK of 0.02 μM in MCF7 and 0.17 μM in SW620 cells) including chemo- and radiosensitization potential and improved drug-like properties compared to NU7441, counter screen against other PIKK family members revealed that this compound as well as the other synthesized analogues were in fact very potent dual DNA-PK and PI3K inhibitors (Cano et al., 2013; Munck et al., 2012). Similarly, the DNA-PK ATP-competitive inhibitor SU11752 was shown to inhibit also PI-3 K p110γ (Ismail et al., 2004) whereas the specific ATM inhibitor KU55933 was reported to bear considerable DNA-PK inhibitory activity (Hickson et al., 2004). Similarly, Hisatomi et al. have suggested in 2011 the topoisomerase IIα inhibitor NK314 as a dual topoisomerase and DNA-PK inhibitory molecule by showing that NK314 potentiates its antitumor activity in adult T-cell leukemia/lymphoma cells by inhibition of dual targets (Hisatomi et al., 2011).

Very recently, Willoughby et al. reported that NU5455, a newly identified highly selective oral DNA-PKcs inhibitor, preferentially augmented the effect of targeted radiotherapy on human orthotopic lung tumors without influencing acute DNA damage or a late radiation-induced fibrosis (Willoughby et al., 2020). Furthermore, while NU5455 administration increased both the efficacy and the toxicity of parenterally administered topoisomerase inhibitor, it enhanced the activity of doxorubicin released locally in liver tumor xenografts without inducing any adverse effects (Willoughby et al., 2020). These findings are of particular importance for the treatment of HCC as in clinical scenarios, local treatments (localized doxorubicin-eluting beads) are being used (Lammer et al., 2010) and DNA-PKcs activity has been reported to confer resistance to these treatments (Cornell et al., 2015). Transient pharmacological inhibition of its activity by NU5455 is effective and tolerable when combined with localized DNA-damaging therapies and thus has promising clinical potential (Willoughby et al., 2020).

4.1.3. IC60211 derivatives

Another class of selective anti-DNA-PK compounds used in preclinical studies comprises molecules from the series of morpholine-containing inhibitors derived by optimization from the arylmorpholine IC60211 (IC_{50} = 400 nM) from the ICOS Corporation small molecule library (Kashishian et al., 2003; Knight et al., 2004; Shinohara et al., 2005) (Fig. 4C). IC86621, IC486154, IC87102 and the most selective IC87361 all maintain the arylmorpholine substructure and are highly potent (IC_{50} = 120 nM, 44, 35 and 34, respectively) (Kashishian et al., 2003; Knight et al., 2004; Shinohara et al., 2005). Although IC86621 with an IC_{50} of 120 nM is not the most active of these compounds, it can be easily synthesized (Chandra et al., 2012), is chemically stable and thus became the most used representative of these inhibitors (Allen et al., 2003; Bailey et al., 2004; Peddi et al., 2010; Yang et al., 2009a, 2009b; Yasaei et al., 2013; Yasaei and Slijepcevic, 2010). IC86621 was shown to enhance cellular toxicity of etoposide and bleomycin but not the one of doxorubicine, cisplatin, 5-FU, paclitaxel or vinblastine, indicating that its activity specifically affects cellular metabolism of DNA DSBs but not of single-stranded DNA breaks or other DNA lesions (Kashishian et al., 2003). Structurally different small molecule DNA-PK inhibitor IC486241, which possesses an acridinone core, was found to synergize with irinotecan to enhance colon cancer cell death (Davidson et al., 2012a, 2012b) and to sensitize breast cancer cell lines to doxorubicin and cisplatin (Davidson et al., 2012a, 2012b).

Importantly, although IC86621 and related compounds all display certain activity against the closely related PI3Ks, they appear benign in the absence of exogenous DSBs at concentrations of up to 50 μM (Kashishian et al., 2003). Nevertheless, with the aim of avoiding the risk of radiosensitization of normal tissue, Wong et al. has recently reported development of SN38023, a novel bioreductive pro-drug that is metabolized to IC87361 selectively in radioresistant hypoxic cells (Wong et al., 2019).

4.2. DNA-PK inhibitors in clinical trials

As outlined in the previous chapter, clinical testing of DNA-PK inhibition has been initially tampered by the lack of specificity and insufficient pharmacokinetic properties of the early inhibitors. The two Celgene drugs, CC-115 and CC-122, were among the first to be evaluated in clinical settings, followed by a pan-PI3K inhibitor ZSTK474 (Zenyaku Kogyo Co., Ltd.) that targets also DNA-PK, and the selective DNA-PK inhibitors VX984 (M9831; Vertex Pharmaceuticals and Merck KGaA), M3814 (EMD Serono Research & Development Institute, Inc.) and AZD7648 (AstraZeneca), currently tested in numerous trials (Table 2; Fig. 4C).

4.2.1. CC-115, a dual DNA-PK and mTOR inhibitor

CC-115 has been synthesized and reported by Mortensen et al. in 2015 as a triazol-containing mTOR inhibitor with pharmacokinetic properties that granted its further clinical development (Mortensen et al., 2015). Later on, Tsuji et al. have reported the characterization of the DNA-PK inhibitory activity of CC-115 *in vitro*, proving that this compound inhibits DNA-PKcs autophosphorylation at Ser2056 and prevents NHEJ by inhibiting the dissociation of DNA-PKcs, XRCC4, and DNA ligase IV from DNA ends (Tsuji et al., 2017). Interestingly, using CC-115 they

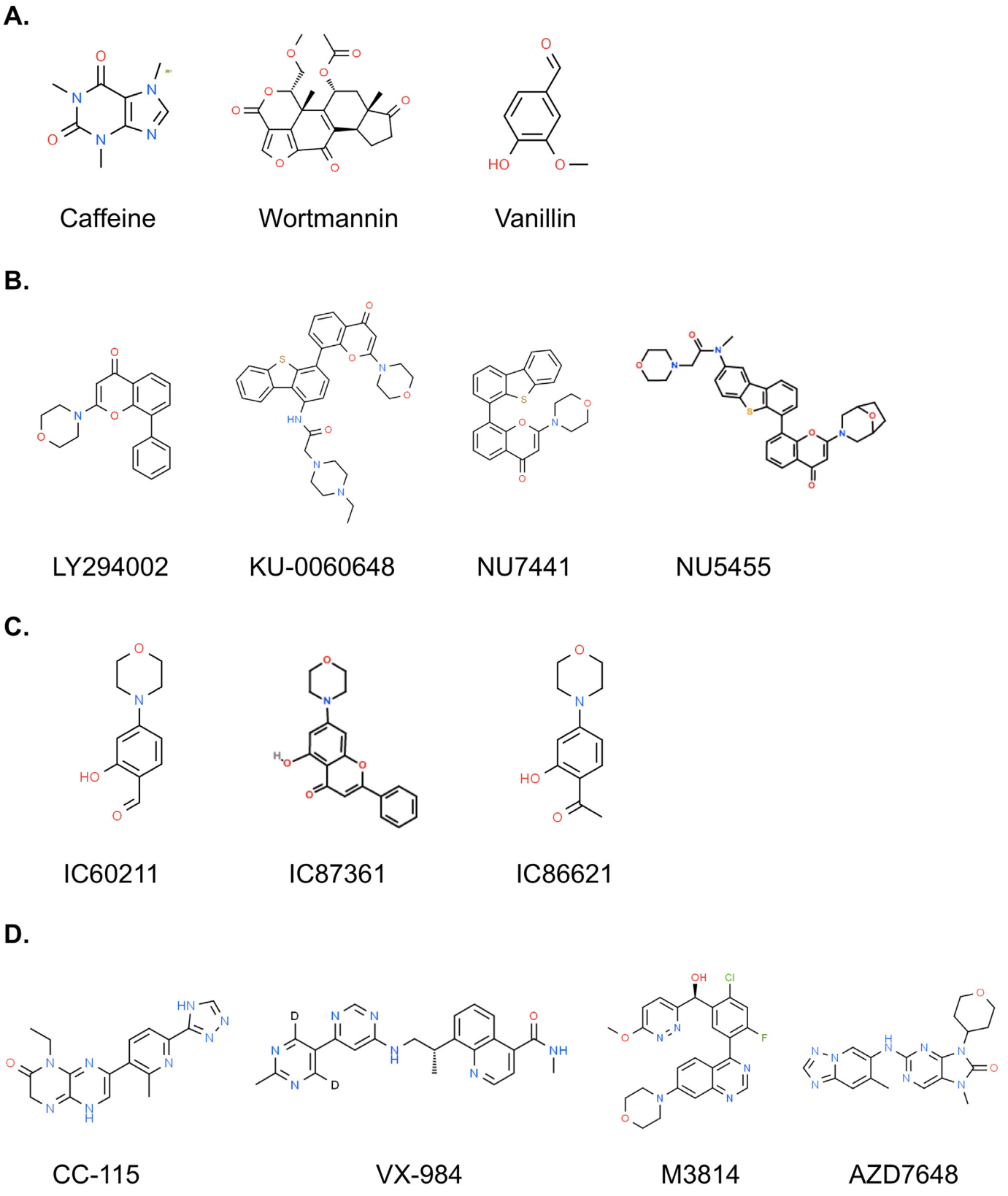

Fig. 4. Chemical structures of representative DNA-PK inhibitors: (A) early compounds, (B) LY294002 and related inhibitors, (C) IC series, (D) compounds in clinical trials (drawings of chemical structures were produced by ChemSpider).

also showed that inhibition of DNA-PK is synthetically lethal with the loss of functional ATM, a finding that would also support CC-115 clinical development in patients with ATM-deficient tumors.

CC-115 as a dual DNA-PK/mTOR inhibitor is a promising compound for management of CLL as in this disease, B-cell receptor (BCR) signaling inhibition provides clinical benefit to patients but acquired resistance

often arises due to mutations in the DNA damage and BCR pathways and preclinical studies have reported encouraging data (Thijssen et al., 2016).

NCT01353625 was the first-in-human Phase I study of CC-115, initiated back in 2011, with a total of 118 enrolled participants. The main purpose of this study was to assess the safety and action of this new class of experimental dual kinase inhibitors targeting DNA-PK and mTOR in patients with advanced tumors (solid and hematologic malignancies) unresponsive to standard therapies and to determine the appropriate dose and tumor types for later-stage clinical trials. Already in 2016, Thijssen et al. have reported promising results concerning early clinical activity of CC-15 in patients with CLL (Thijssen et al., 2016). BCR-mediated signaling was inhibited by CC-115 in CLL samples obtained from patients with acquired resistance to the approved PI3K inhibitor idelalisib (Thijssen et al., 2016). Clinically, 7 out of 8 evaluated study participants with relapsed/refractory CLL/small lymphocytic lymphoma that harbored mutations or deletions in the *ATM* gene had a decrease in lymphadenopathy, resulting in 1 partial response (PR) and 3 PRs with lymphocytosis (Thijssen et al., 2016).

More recently, a study on dose-finding and cohort-expansion phases (74 patients) of the NCT01353625 trial has revealed that CC-115 was well tolerated and the observed toxicities were consistent with those reported for mTOR inhibitors (Munster et al., 2019). These included in the dose-finding cohort (44 patients) thrombocytopenia, stomatitis, hyperglycemia, asthenia/fatigue and increased transaminases whereas in the cohort expansion (74 patients, 5 specified tumor types), for which CC-115 10 mg BID was selected, fatigue, nausea, and decreased appetite were the most frequently observed (Munster et al., 2019).

Concerning preliminary efficacy of CC-115, a patient with endometrial carcinoma remained in complete remission for more than 4 years and 38% and 25% of CLL/small lymphocytic lymphoma patients had PR and stable disease (SD), respectively (Munster et al., 2019). SD was also reached in 53%, 22%, 21%, and 64% of patients with HNSCC, Ewing sarcoma, GBM, and castration-resistant prostate cancer, respectively (Munster et al., 2019). Out of twelve additional patients with mixed solid tumors that participated in a bioavailability substudy, 2 experienced PR and 4 SD (Munster et al., 2019).

Currently, two other studies, NCT02833883 and NCT02977780, are assessing the dual DNA-PK and mTOR inhibition potential of CC-115 in clinical settings. NCT02833883 is a Phase 1b study combining CC-115 with the nonsteroidal antiandrogen enzalutamide for treatment of men with castration-resistant prostate cancer. Results of this trial, which has been initialized in July 2016 at Memorial Sloan Kettering Cancer Center and has recruited 40 participants, are expected to be available by July 2021. The prime purpose of the INdividualized Screening Trial of Innovative Glioblastoma Therapy (INSIGhT) study (NCT02977780) led by Patrick Y. Wen (Dana Faber Cancer Institute) is to evaluate various investigational drugs such as abemaciclib (a Cdk4/6 inhibitor), neratinib (a dual HER2/EGFR tyrosine kinase inhibitor) and CC-115 as a possible treatment for GBM patients. INSIGhT estimates recruitment of 280 participants and will be concluded by mid-2022.

Concerning potential resistance mechanisms towards CC-115 treatment, Beebe and Zhang have reported in their recent preclinical study that CC-115 is a substrate of ATP-binding cassette G2 (ABCG2) and suggested that expression of ABC transporters, including ABCB1 and ABCG2, may affect the outcome in clinical trials testing CC-115 (Beebe and Zhang, 2019). Moreover, their data indicate that ABC transporters may be used as markers for future precision use of CC-115.

4.2.2. CC-122 and ZSTK474 as non-selective DNA-PK targeting

Avadomide (CC-122), a pleiotropic pathway modifier compound originally developed for broad diffuse large B-cell lymphoma (DLBCL) and with activity against DNA-PK (Goodwin and Knudsen, 2014; Trenner and Sartori, 2019), is studied in Phase I first-in-human study in patients with advanced solid tumors, non-Hodgkin's lymphoma (NHL), or multiple myeloma (NCT01421524; 271 participants). In three DLBCL patients included in this trial, CC-122 was shown to regulate the natural killer cells phenotype and its activity due to the reduced accumulation of myeloid-derived suppressor cells and eventually decrease the T regulatory lymphocytes subsets and the activation of T cells through co-stimulatory molecule CD28 was detected as a delayed CC-122 effect (Cubillos-Zapata et al., 2016). Later on, Rasco et al. reported that avadomide monotherapy demonstrated acceptable safety and favorable pharmacokinetics in these patients, with 3 objective responses observed for NHL cases (Rasco et al., 2019). Furthermore, among 84 patients with *de novo* relapsed or refractory DLBCL, overall response rate to this drug with immunomodulatory and direct antitumor activities was 29%, including 11% of complete responses (Carpio et al., 2020). Avadomide is currently being evaluated for various indications in combination with other therapeutics such as the checkpoint inhibitor nivolumab in unresectable HCC and advanced melanoma. However, as CC-122 is a small-molecule therapeutic agent that primarily modulates the cereblon E3 ligase activity, its effects as an anti-DNA-PK compound are rather difficult to estimate.

Similarly, ZSTK474 (Zenyaku Kogyo Co., Ltd.), a pan-PI3K inhibitor that to some extent inhibits also DNA-PK (Kong and Yamori, 2010), has been tested in two phase I clinical trials (NCT01280487 and NCT01682473) for advanced solid malignancies. Plausible contribution of its DNA-PK-targeting properties to the overall treatment efficacy is however unknown.

4.2.3. VX984 (M9831), M3814 and AZD7648: specific DNA-PK catalytic inhibitors

VX984 (M9831; Vertex Pharmaceuticals, Merck KGaA) is a catalytic DNA-PK kinase inhibitor, which was shown to efficiently inhibit NHEJ, resulting in compensatory increases in alternative repair pathways, preferentially in transformed cells (Khan et al., 2018). When evaluated in combination with irradiation in preclinical GBM models, enhanced radiosensitivity was observed *in vitro* as well as in orthotopic xenografts, indicating that this compound crosses the blood-brain barrier at sufficient concentrations (Timme et al., 2018). VX984 has been tested clinically solely in the first-in-human phase I trial (NCT02644278), which has been initiated in the US in December 2015. The purpose of this study was to evaluate the safety and tolerability of VX984 administered alone and in combination with PLD, and to determine the maximum tolerated dose (MTD) and preliminary evidence of efficacy of VX984 in combination with PLD in participants with advanced solid tumors. However, the study with 15 enrolled participants has been prematurely discontinued during the dose escalation part based on business-related reasons.

M3814 (MSC2490484A, nedisertib, peposertib; Merck KGaA) is an investigational drug that is being evaluated for the treatment of subjects with advanced solid tumors or CLL that likely differs from other cancers in the mode of DNA repair. *In vitro*, M3814 sensitized multiple tumor cell lines to radiation therapy and strongly enhanced the antitumor activity of ionizing radiation *in vivo* with complete tumor regression upon fractionated radiation (Zenke et al., 2020). These effects are due to inhibition of DNA-PK protein kinase activity as demonstrated by the levels of DNA-PK autophosphorylation in human tumor cell lines, and xenograft tumors (Zenke et al., 2020).

Recent preclinical studies have reported activity of M3814 in combinations with other therapeutic approaches in various tumor types. In this respect, Klein et al. have shown in 2017 that DNA-PK inhibition by M3814 combined with either photon or carbon ion irradiation overcomes hypoxia-induced radioresistance in NSCLC models (Klein et al., 2017). More recently, Wise et al. have evaluated the activity of M3814 in combination with multiple topoisomerase II inhibitors in ovarian cancer xenografts (Wise et al., 2019). In these models, M3814 had only a limited efficacy as a monotherapy but in combination with PLD, enhanced activity has been observed as compared to PLD treatment alone. Importantly, M3814 has been well tolerated. In the context of acute myeloid leukemia, the 2020 data by Carr et al. propose M3814

as a new combination partner of Mylotarg, the first AML-targeting drug from a new generation of antibody drug conjugate therapies aiming with increased specificity at the acute leukemia cell compartment (Carr et al., 2020).

Mechanistically, Sun et al. have reported that M3814 effectively blocks the repair of radiation-induced DSBs and potently enhances p53 phosphorylation and activation (Sun et al., 2019). Interestingly, whereas upon the combined M3814/irradiation treatment the p53 wild-type cells underwent a complete p53-dependent cell cycle arrest and premature cell senescence, p53-deficient cell lines experienced mitotic catastrophe and apoptotic cell death due to cell cycle progression despite a DNA damage load (Sun et al., 2019). These data identify p53 as a possible biomarker for response of cancer cells to combination treatment with radiation and a DNA-PK inhibitor and suggest that p53 mutation status should be considered in the design of future clinical testing.

M3814 has been initially tested in two phase I clinical trials as monotherapy (NCT02316197; 31 participants) or in combination with (chemo-)radiotherapy (NCT02516813; 155 participants). In both trials patients were treated with ascending doses of M3814 to establish the safety and maximum tolerated dose (MTD) or recommended phase 2 dose (RP2D) (primary endpoint) and efficacy (secondary endpoint). Based on preliminary results, M3814 has been well tolerated as monotherapy (most frequent adverse events in NCT02316197 were nausea, vomiting, decreased appetite, constipation, diarrhea, pyrexia, fatigue, and rash, whereas in NCT02516813 (first 16 patients), dysphagia, prolonged mucosal inflammation/ stomatitis and radiation skin injury) and the RP2D of 400 mg BID has been selected (Mau-Sorensen et al., 2018). Pharmacodynamics in surrogate tissue showed robust inhibition of the induction of phospho-DNA-PK inhibition for up to 6 h and whereas no objective responses were reported in the monotherapy trial, while in the combined settings (M3814 administered 1,5 h prior IR/IR + cisplatin), durable in field responses were seen in 7 out of 16 evaluated patients (Mau-Sorensen et al., 2018).

Following these two trials, numerous other studies evaluating M3814 in combination with other therapies are currently ongoing. Among them is the phase I study testing avelumab-M3814 combinations with and without radiotherapy in participants with selected advanced or metastatic solid tumors (NCT03724890; recruiting, estimated 34 participants), the phase I/II study of M3814 in combination with capecitabine and radiotherapy in locally-advanced rectal cancer (NCT03770689; recruiting, estimated enrollment 160 patients), NCI-sponsored trials NCT03983824 (M3814 in combination with Mitoxantrone, Etoposide, and Cytarabine for Relapsed or Refractory Acute Myeloid Leukemia), NCT04172532 (a phase 1/2 Study of M3814 in combination with hypofractionated radiotherapy for the treatment of unresectable locally advanced pancreatic adenocarcinoma), NCT04092270 (a phase I/Ib dose escalation study of pegylated liposomal doxorubicin with M3814 in ovarian cancer), NCT04071236 (a phase I/II study of Radium-223 Dichloride, M3814 and avelumab for Advanced Prostate Cancer Not Responsive to Hormonal Therapy), NCT04068194 (a phase I/II study of M3814 and avelumab in combination with hypofractionated radiation in patients with advanced/metastatic solid tumors and hepatobiliary malignancies) as well as the NCT04266912 phase I/II study aiming at the evaluation of avelumab and M6620 or M3814 (nedisertib) for the treatment of DDR-deficient metastatic or unresectable solid tumors.

Early this year, Goldberg et al. reported AZD7648 as a very potent and highly selective DNA-PK inhibitory molecule that resulted from a screen of AstraZeneca's corporate collection for DNA-PKcs inhibitors with good PI3K selectivity (Goldberg et al., 2020). Optimization of the best hit focused on further increase in selectivity while improving physical and pharmacokinetic properties such as co-optimization of permeability and metabolic stability. AZD7648 did not display any significant off-target activity in the protein kinome and only weak activity *versus* PI3Kα/γ lipid kinases. Monotherapy activity in murine xenograft models was observed.

Concerning combinations with relevant anticancer therapies, AZD7648 sensitized xenografts and patient-derived xenografts to radiation- and doxorubicin-induced DNA damage and induced sustained regressions in these models (Fok et al., 2019). In addition, AZD7648 was efficient also in combinations with the PARP inhibitor olaparib by increasing genomic instability, resulting in cell growth inhibition, apoptosis and sustained tumor regressions.

Based on the promising preclinical findings, AZD7648 has progressed into clinical testing. NCT03907969 is a modular Phase I/IIa clinical trial to evaluate AZD7648 alone and in combination with other anticancer agents such as doxorubicin or olaparib in patients with advanced solid cancers. The study, which is currently recruiting participants (estimated enrollment of 234 patients), is expected to be concluded early in 2024.

5. Concluding remarks and perspectives

The fundamental role that DDR effectors play in cell physiology by preserving genome stability, thereby averting malignant transformation, has become an established dogma during recent decades. Consequently, and with the introduction of PARP inhibitors as a routine pharmacological approach in the case of BRCA1/BRCA2 deficient tumors, the repertoire of additional DDR targets that are aberrantly expressed in cancer, is expected to steadily expand in the near future to come (Yap et al., 2019). A host of preclinical and clinical findings has been accumulating that suggests that both a decrease and an increase in DNA-PK status and activity is a characteristic present in numerous tumors and tumor models. In the emerging era of precision medicine, we foresee that it will be of utmost importance to apply patient stratification parameters in order to identify and select the patients who could benefit from DNA-PK inhibitors that are already available and the next generations compounds, which will appear in the upcoming future. To that end, much work is probably still required where the biology of particular genomic alterations is still not optimally clarified as for example in the case of the various Ku70, Ku80 and DNA-PKcs SNPs. In other words, it will be essential to determine in this instance the relevance of a particular DNA-PK variant found in a tumor for the treatment with a particular DNA-PK inhibitor. To that end, there is currently still a gap with preclinical studies that should clarify the biology of those polymorphism variants to explain their particular association with patient prognosis.

An important aspect of the use of DNA-PK inhibitors in the clinic is the determination of a full broad of combinations with other treatment modalities, which could deliver optimal treatment results. In that context, combining those inhibitors with any DDAs in addition to RT could be of a therapeutic benefit in terms of tumor sensitization. Obviously, expanding such combined treatment also to other modalities as specific kinase inhibitors and immunotherapies could translate into efficient next generation protocols.

Importantly, DNA-PK targeting could also be investigated in the context of HR deficiency as with BRCA1/BRCA2 deficient breast, ovarian and pancreatic cancers in combination with PARP inhibitors. The corresponding additional blocking of NHEJ in these tumors with another DDAs intervention could have an added therapeutic impact that needs to be determined.

Obviously, targeting DNA-PK is predominantly likely to have sensitizing effects to DDAs in tumors with increased NHEJ activity. In tumors exhibiting decreased DNA-PK activity, a strategy that would target HR factors is worthwhile to be explored. An additional path that has not been yet explored effectively are screenings, using CRISPR or pharmacologic libraries, for identification of targetable proteins that could form synthetic lethal interactions with DNA-PK (Kantidze et al., 2018).

The emerging biology of the DNA-PK signaling programs out of the DDR is another tremendously important feature that without doubt could be extended to additional tumor types beyond prostate cancer. The fact that an originally-defined DDR effector regulates networks that control tumor metastasis and that its inhibition can interfere with this attribute of tumor progression are remarkable findings with game changing potential. It would be certainly of utmost importance to reveal whether interphases similar to the one between the AR and DNA-PK in prostate cancer exist and are relevant to other hormone receptors that control tumorigenesis of other tissues. Obviously, the significance of such questions would foremost apply to the ER and PR in breast cancer.

Notably, during the last two decades, interesting findings that point to a signaling crosstalk between the DDR and growth factor receptor tyrosine kinases, which are major cancer drivers have been reported. The interplay between pathways driven by the EGFR and DNA-PK is of relevance as discussed above. Naturally, it would be of high interest to further determine whether other RTKs may have similar interactions with the DNA-PK complex and how inhibition or co-inhibition of those receptors with DNA-PK targeting may affect various tumor features as growth and responses to DDAs.

Finally, as resistance mechanisms ultimately evolve with almost any targeting approach, it is probably fair to speculate that this will happen also when DNA-PK inhibitors will find the way to the clinic. Identifying potential resistances to the DNA-PK inhibitors in preclinical setting might, as it has been shown in other cases, to be beneficial for patients.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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