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DNA Damage Response Pathways and Cell Cycle Checkpoints in Colorectal Cancer: Current Concepts and Future Perspectives for Targeted Treatment

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Abstract

Although several drugs have been designed in the last few years to target specific key pathways and functions in colorectal cancer (CRC), the backbone of CRC treatment is still made up of compounds which rely on DNA damage to accomplish their role. DNA damage response (DDR) and checkpoint pathways are intertwined signaling networks that arrest cell cycle, recognize and repair genetic mistakes which arise during DNA replication and transcription, as well as through the exposure to chemical and physical agents that interact with nucleic acids. The good but highly variable activity of DNA damaging agents in the treatment of CRC suggests that intrinsic alterations in DDR pathways and cell cycle checkpoints may contribute differentially to the way cancer cells react to DNA damage. In the present review, our aim is to depict the recent advances in understanding the molecular basis of the activity of DNA damaging agents used for the treatment of CRC. We focus on the known and potential drug targets that are part of these complex and intertwined pathways. We describe the potential role of the checkpoints in CRC, and how their pharmacological manipulation could lead to chemopotentiation or synergism with currently used drugs. Novel therapeutic agents playing a role in DDR and checkpoint inhibition are assessed. We discuss the possible rationale for combining PARP inhibition with DNA damaging agents, and we address the link between DDR and EGFR pathways in CRC.

Keywords

Colorectal cancer; Chk2; cell cycle; DNA damage response; irinotecan; oxaliplatin

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CONFLICT OF INTEREST

None.

INTRODUCTION: COLORECTAL CANCER, DNA DAMAGE RESPONSE PATHWAYS, AND THE CELL CYCLE CHECKPOINTS

Replication of DNA by living organisms requires order and control to ensure correct transmission of all the information coded by nucleic acids from one generation to the next. Indeed, since the first self-reproducing nucleic acids appeared on the Earth, in the likely shape of RNA molecules [1, 2], billions of years of evolution have contributed to the development of one of the most precise and complex machineries of the cell: the DNA damage response (DDR) pathways.

Broadly speaking, DDR pathways are intertwined signaling networks that recognize and repair mistakes that unavoidably arise during DNA replication and transcription, as well as through the exposure of living beings to natural and artificial chemical and physical agents that interact with nucleic acids, damaging them and endangering their fundamental function, i.e. propagating correct genetic information across generations of organisms (prevention of germinal mutations), and across generations of cells inside the same multi-cellular organism [3]. Within the DDR pathways, the eukaryotic cell possesses checkpoint control mechanisms that can arrest the various phases of cell replication and allow for the repair of DNA damage (either endogenously generated by replication itself or through exogenous agents) [4–7]. These processes determine whether the cells repair their genetic material, thus completing the synthesis of sister chromatids and mitosis, or undergo programmed cell death (apoptosis).

Bert Vogelstein defined cancer as “a genetic disease of the somatic cell” [8]. Such a synthetic definition is particularly appropriate to define the genesis of colorectal cancer (CRC). Through either chromosomal instability (CIN, possibly mediated by APC (adenomatous polyposis coli) loss of function or by other mechanisms [9]) or abnormal accumulation of mutations (such as in mismatch repair (MMR) deficiency [10, 11]), colon mucosa cells bearing DNA alterations may survive and proliferate, eventually accumulating enough aberrancies in key genes to allow them to duplicate without control, escape normal cell-cell and cellmatrix inhibitions, acquire an aberrant behavior, and generate tumors.

CRC carcinogenesis is possibly one of the best studied and characterized pathological phenomena in humans [12]. This is due in part to the relative easiness of detection and removal of early, pre-cancerous lesions through instrumental procedures, which has allowed the identification of sequential phases of CRC development. Also, familial clusters of CRC have provided insights into the function of specific genes involved in CRC development, the loss of which results in CRC with extremely high frequency compared to subjects that do not bear such mutations (see Table 1). The germline-mutated genes which charachterize these syndromes are often part of DDR pathways, or directly influence chromosomal stability: emblematic cases are the association of APC/Wnt pathway function loss with CIN [13, 14], and that of MMR deficiency with microsatellite instability and the so called “mutator phenotype” (*i.e.*, a cancer characterized by an increased mutation rate compared to healthy tissues) [15, 16]. Although several drugs have been designed in the last few years to target specific key pathways and functions in CRC (see Bagnasco L. *et al.,* and Ballestrero A. *et al.,* in the present issue of CCDT), the backbone of CRC treatment is still made up of

compounds which rely on DNA damage to accomplish their role. Such agents (irinotecan, oxaliplatin, and 5-fluorouracil) yield highly variable, but often important results in patients affected by CRC. This suggests that intrinsic alterations of CRC subtypes may contribute differentially to the way cancer cells react to DNA damage. Indeed, both familial and sporadic CRC is often characterized by aberrancies in DDR pathways (resulting in increased chromosomal or microsatellite instability) and these may explain, in part, such phenotypes of sensitivity/resistance.

Here, we review the best characterized mechanisms that, in CRC, confer susceptibility/resistance to two DNA damaging chemotherapeutics that are commonly used for the treatment of CRC, irinotecan and oxaliplatin. We describe the potential role of checkpoint pathways, the manipulation of which may lead to chemopotentiation or synergism with currently used drugs, and we discuss the possible rationale for combining PARP inhibition with DNA damaging agents in CRC. The intertwining of DDR pathways with EGFR pathway activation is also addressed. We will not deal with the issue of sensitivity/resistance of CRC to 5-fluorouracil (5-FU), because of the incompletely ascertained relative role of DDR and checkpoint pathways in the response to this agent, compared to its more widely studied mechanism of thymidylate synthase blocking and RNA synthesis interference. We refer the reader to in-depth reviews dealing with such topic (see [17–19]).

PHENOTYPES OF SUSCEPTIBILITY/RESISTANCE TO DNA DAMAGING AGENTS IN CRC: IRINOTECAN AND OXALIPLATIN

Irinotecan

Irinotecan (Camptostar®, Pfizer Inc.) is a semisynthetic analogue of camptothecin (CPT) [34], an anticancer compound isolated from the barks of *Camptotheca Acuminata*. CPT was discovered in 1966 during systematic *in vitro* screenings with plant-derived compounds [35]. Camptothecins are topoisomerase I (Top1) inhibitors, acting as interface traps between the enzyme Top1 and DNA, and stabilizing such ternary macromolecular complex. As a consequence, DNA double-strand breaks (DSBs) are generated, and massive DNA damage ensues [34]. Following two large phase III clinical trials, irinotecan was approved as first line treatment of CRC, in combination with 5-FU and leucovorin [36, 37]; it is also active in several other types of solid tumors [38, 39]. Irinotecan is scarcely active *per se*, but needs to be activated into the highly potent metabolite SN-38 through enzymatic hydrolysis by carboxylesterase 1 (*CES1*) [40], *CES2* [41], and butyrylcholinesterase (*BCHE*) [42]. These enzymes are widely expressed in tissues. On the other hand, inactivation of SN-38 occurs through glucuronidation into the inactive compound SN-38G by liver UDP glucuronosyltransferases 1A1, 1A7 and 1A9 (*UGT1A1*, *UGT1A7*, and *UGT1A9*) [43–47], and elimination *via* biliary fluids of irinotecan and its metabolites is due to efflux pumps like ABCB1 and ABCB2 (irinotecan, SN-38, and SN-38G), as well as ABCG2 (SN-38) [48–51]. Needless to say, several studies have assessed the association of variants and polymorphisms of the aforementioned genes with the high variability in activity and toxicity of irinotecan in clinical practice. As a result, the *UGT1A1*28* genotype is now accepted as a predictor of irinotecan toxicity, and the FDA recommends its testing before starting irinotecan-containing regimens [52]. Likewise, an *ABCG2* single nucleotide polymorphism (SNP) has been

associated with increased myelosuppression upon irinotecan-based regimen administration [53]. Clearly, prospective studies assessing multiple variables that may confer predisposition to increased toxicity or to diminished activity of irinotecan are warranted to establish a panel of recognized predictors of toxicity that this compound may cause in patients. Apart from variability in the genes that mediate the pharmacokinetic response to irinotecan, other cancer-cell specific factors must play a role in determining the activity of Top1 inhibitors. This can be evinced by the high variability of CPT toxicity in large *in vitro* screenings, like those performed in the NCI-60 panel of cancer cell lines [54]. Although this *in vitro* variation is due, in part, to the overexpression of efflux pumps by cancer cells, the noncamptothecin-like Top1 inhibitors (*e.g.*, the indenoisoquinolines [55]), which are not multi-drug resistance pump substrates, exhibit variability in their *in vitro* activity. By themselves, compounds like irinotecan do not create irreversible complexes with Top1 and DNA. Only when advancing replication forks collide with such macromolecular complexes, there is generation of DSBs, with activation of DDR, cell cycle block, and cell death. *TOP1* mRNA and protein expression levels have been demonstrated to correlate directly, *in vitro*, with the activity of Top1 inhibitors [56]. Dosing Top1 levels in cancer may therefore predict irinotecan response. Finally, several enzymes contribute to Top1–DNA complex removal and DNA integrity preservation. Tyrosyl-DNA phosphodiesterase (TDP1) hydrolyzes the phosphodiester bond at a DNA 3'-end linked to a tyrosyl moiety and has been implicated in the repair of Top1-DNA covalent complexes. TDP1 possesses the ability to hydrolyze and remove other 3' DNA lesions, like 3' abasic sites and 3' phosphodycolate, suggesting its activity as a DNA repair enzyme [57]. Thus, inhibiting TDP1 may synergize with Top1 inhibition or, alternatively, TDP1 overexpression may be a resistance factor to Top1 inhibition in cancer cells [58]. Likewise, siRNA-silencing of *ERCC1* (excision repair cross complementation group 1), an endonuclease involved in nucleotide excision repair (NER), was recently shown by our group to potentiate the effects of CPT in concomitance with the blockade of PARP by Veliparib (Abbott, Inc.) [59], a PARP1/2 inhibitor currently undergoing clinical trials (http://clinicaltrials.gov). Clearly, DDR pathways play an important role in determining cancer sensitivity to Top1 inhibitors. On the one hand, knowing the presence of alterations in DDR components, *via* either somatic mutations, or expression modifications may be useful in predicting the response of CRC to irinotecan. On the other hand, such gene products may be susceptible to selective inhibition with the rationale of potentiating the effect of Top1 inhibitors.

Oxaliplatin

Oxaliplatin was discovered in 1976 at Nagoya City University, in the effort of finding water-soluble platinum-based compounds that could have good antitumor activity, yet lacking cross-resistance with cisplatin [60]. Following the observation of high activity against *in vitro* models of CRC [61], and of the synergistic effect of combining oxaliplatin with 5-FU *in vitro* and *in vivo* [62], great efforts were placed in the clinical development of this compound in patients affected by CRC [63]. The combination of oxaliplatin with 5-FU and leucovorin was then confirmed as a valuable treatment option for CRC in phase III studies conducted in patients with advanced disease [64, 65] and in the adjuvant setting of lymph-node positive, non-metastatic CRC [66, 67]. Moreover, second-line infusional 5-FU, leucovorin, and oxaliplatin (FOLFOX4) was recently shown to be non-inferior to irinotecan

in metastatic CRC, with a better time-to-progression and response rate and fewer grade III side effects than irinotecan alone [68]. Oxaliplatin is now approved for stage III and metastatic CRC, in combination with 5-FU and leucovorin; the addition of the antiangiogenetic monoclonal antibody bevacizumab to this drug combination has led to even better results in terms of overall survival in patients with metastatic CRC (see Bagnasco L., *et al.,* in the present issue of CCDT). Although exhibiting different spectra of activity, for only partially understood reasons [63, 69, 70], platinum coordination complexes are thought to act mainly by covalently binding to nucleophilic sites on cellular DNA (especially with the N7 position of guanine – GG intrastrand crosslinks), *via* inter- and intrastrand crosslinks, and DNA-protein crosslinks [71]. Therefore, these compounds prevent cell division and growth [71], and disrupt vital processes like DNA replication and transcription [72], finally leading to cell death by apoptosis [69, 73]. Like irinotecan, pharmacokinetics of oxaliplatin influence its activity (see Fig. 1). Platinum compounds enter the cell through an active copper transporter, SLC31A1 [74], and are actively expelled by cytoplasm member transporters (ABCC2 and ABCG2 [75–78]) and through trans-Golgi network copper pumps (ATP7A and ATP7B [79, 80]). While increased expression of such efflux pumps has been repeatedly associated with resistance to platinum-base compounds, SLC31A1-mediated influx of oxaliplatin is probably among the determinants of the ototoxicity observed in oxaliplatin treated patients [81]. Also, several genes involved in detoxification processes (e.g., MPO, SOD1, GSTM1, NQO1, GSTP1, and MT), are able to lower the concentration of platinum-based compounds inside the cell, and specific alleles of these genes may contribute to differences in sensitivity and toxicity observed among treated patients [71].

A unique feature of oxaliplatin, compared to cisplatin, is its ability to form bulkier adducts to DNA, and such lesions differ from those induced by cisplatin concerning patterns of distortion of the DNA helix, and of hydrogen bonding to neighboring DNA segments [82]. Such oxaliplatin properties may explain in part its different spectrum of activity compared to other compounds of the same class. Indeed, oxaliplatin-GG adducts possess distinct minor conformations compared to cisplatin-GG ones [83]. As a consequence, high-mobility group proteins like HMGB1, which recognize platinum-DNA adducts and stimulate DDR response [84], show smaller binding affinity to oxaliplatin-GG adducts and are probably less important in determining the response of cancer cells to its toxic effects [83]. Once platinum adducts are established, the MMR and the NER pathway are involved in the subsequent management of the generated lesions, while translesional replication polymerases can proceed through DNA lesions and continue DNA replication (see Fig. 1). Intrguingly, while non-functional NER components (in particular polymorphisms of *ERCC1*, *XPS*, and *XRCC1* genes), which repair bulky DNA lesions, are associated with increased sensitivity of CRC to platinum-based compounds [85–87], overexpression of members of NER pathways such as *ERCC1* is associated with poor response to platinum-based chemoradiotherapy in non-smallcell lung cancer [88]. The link between *ERCC1* expression and poor response to oxaliplatin-based chemotherapy has also been reported in CRC [89]. In contrast with cisplatin, oxaliplatin-mediated activity does not seem to depend on a functional MMR pathway to induce cell apoptotic response [63, 69, 90]: this aspect of oxaliplatin-mediated toxicity may explain its activity in CRC, where the MMR pathway members are thought to be deficient in up to 15% of sporadic CRC [91, 92]. Finally, the greater efficiency of bypass

of oxaliplatin-GG adducts by translesional replication polymerases like POLH may in part explain the better mutagenicity profile of oxaliplatin *versus* cisplatin [70], but increases in replicative bypass, which may play a role in cisplatin resistance [93], have not been shown to induce such phenotype toward oxaliplatin [69]. In conclusion, both pharmacokinetics (i.e., host and tumor-specific properties) and pharmacodynamics (in our case, tumor-specific properties) can contribute to sensitivity/resistance to oxaliplatin in CRC patients. Anyway, DDR pathways, in particular the NER complex, are likely a key element in determining such phenotypes in CRC.

CELL CYCLE CHECKPOINTS AND CRC

Cellular molecular surveillance systems, defined checkpoints, allow the detection of damaged DNA and temporarily arrest cell cycle progression to enable DNA repair and prevent further damage. This phenomenon provides the rationale to inhibit checkpoints in order to limit the time available for repair and consequently increase the sensitivity of cells to DNA damaging agents [94].

Overview

The cycle of DNA replication, chromosome separation and division is regulated by mechanisms that maintain the order of these steps and ensure that each step is orderly carried out. Key to these processes are the cell cycle checkpoints, where cells may arrest their replication to ensure that DDR mechanisms have time to operate prior to continuing through the cycle into mitosis [95–97]. The G1/S checkpoint is regulated by checkpoint kinase 2 (Chk2) and p53 and the intra-S and G2/M checkpoint is controlled by checkpoint kinase 1 (Chk1*)* (see Fig. 2). The ATM (ataxia telangiectasia mutated)-Chk2 pathway is mainly activated by DSBs produced by ionizing radiations or DNA damaging agents, but can also be activated by replication-mediated DSBs [98]. The ATR (ATR and Rad3 homolog)-Chk1 pathway is mainly activated in the case of replication-mediated DSBs [98]. Checkpoint kinases play a key role in maintaining the integrity of the genome [99]. In cancer cells, defects in the checkpoint proteins and checkpoint control mechanisms are frequent [100] and responsible for tumorigenesis. Loss of the checkpoints may be a cause of genomic instability in cancer cells [101]. Tumors exhibit three kinds of chromosomal alterations: rearrangements in the structure of individual chromosomes, whole chromosome aneuploidy, and the generation of polyploid cells [101].

Several aberrations in the cell cycle checkpoints have been shown to have prognostic significance in CRC [102]. First, we will list the various alterations in the cell cycle checkpoints encountered in CRCs. Then, we will give an overview of the potential utility to target the remaining functional checkpoints for treatment purposes.

Alterations in the Cell Cycle Checkpoints

Mitotic Checkpoints—Mitotic checkpoint deficiencies can contribute to CIN and tumor formation in mammals [103]. Mice with deficiencies in the mitotic checkpoint proteins MAD2, BubR1 and Bub3 present CIN, and some of them have susceptibility to cancer [104–107]. BubR1$^{+/-}$ mice present an increase of incidence of colon adenocarcinomas when

exposed to carcinogens [105]. In most CRCs, a CIN leading to an abnormal chromosome number, aneuploidy, is consistently associated with the loss of function of a mitotic checkpoint [108, 109]. One mutation of *BUB1B* (encoding BubR1) in an aneuploid CRC was a somatic deletion, resulting in the removal of the kinase domain. Such alteration was similar to the truncating mutations found in mosaic variegated aneuploidy (MVA) disease, a rare recessive disease characterized by mutations in *BUB1B* [110]. Lengauer and Wang observed that the spindle checkpoints genes *BUB1* and *BUB1B* are mutated in some CIN tumors of individuals with CRC [111]. Moreover, other studies in CRC demonstrated the presence of mutations in established or putative mitotic checkpoints genes such as *BUB1*, *ZW10*, *ZWILCH* and *KNTC1* (a.k.a. Rod) [112–114]. Even though these mutations occur rarely in CRCs, nonmutational reduction in the expression of such genes has been reported in a small percentage of colorectal tumors, and increase in expression has been reported as well in several CRCs [113, 115–117]. In CRC biopsies obtained from 16 patients, Menssen *et al.,* observed that elevated expression of c-*MYC* correlated with increased MAD2 levels [118]. c-MYC delays prometaphase by direct transactivation of MAD2 and BubR1, and this may have potential implications in the origin of CIN [118]. Taxanes, which disrupt microtubule function and inhibit mitosis [119], have failed to demonstrate significant clinical benefit in phase II trials in CRC [120]. The high incidence of CIN in this disease, coupled with alterations in spindle checkpoint regulators *in vivo*, may explain the disappointing results associated with such category of anti-cancer drugs [120].

CHEK1—The presence of a nucleotide stretch of nine consecutive nucleotides in the coding region of *CHEK1* led to the hypothesis that tumors with defects in MMR and hence with microsatellite instability could accumulate mutations in the *CHEK1*-coding locus. Indeed in CRC, in which microsatellite instability is a frequent event, insertion/deletion of one nucleotide in *CHEK1* coding region could be found [100, 121, 122], resulting in Chk1 protein truncation and inactivation. The resulting truncated Chk1 proteins are predicted to be defective due to the lack of the C-terminal end of the catalytic domain and the complete loss of the SQrich regulatory domain [123]. Bertoni *et al.,* showed that 1 of 10 CRCs with high frequency of microsatellite instability carry the (A)9 *CHEK1* mutation resulting in a truncated protein [122, 124]. Furlan *et al.,* reported this same incidence of *CHEK1* mutations in an independent cohort [121].

CHEK2—The checkpoint kinase 2 gene (*CHEK2*), identified as a breast cancer susceptibility gene, could also be a candidate gene for CRC susceptibility. Liu *et al.,* screened for mutations of all 14 exons of *CHEK2* in 56 CRC cell lines [125]. *CHEK2* mutation in CRC was a low frequency event. Ten cell lines out of the 56 analyzed had sequence variations within the exons, and only DLD-1/HCT-15 showed a heterozygous missense mutation, R145W [125]. Based on sequence analysis, DLD-1/HCT-15 was found to carry one mutant allele (R145W) and one wild-type allele of *CHEK2* [126]. The R145W mutation, which was in the forkhead homology-associated (FHA) domain keeps some basal kinase activity, but cannot be phosphorylated by ATM and cannot be activated following DNA damage. Because the FHA domain is involved in protein-protein interactions, this mutation could affect the association of Chk2 with other proteins [127]. Using next-generation sequencing and targeted resequencing across the panel of 60 established cancer

cell lines from the NCI Anticancer Screen (NCI60), we confirmed the two point mutations, R145W (see above) and A247D (which disrupts a residue in the catalytic motif), in the HCT-15 cell line [128]. Recently, the incidence of the *CHEK2* 1100delC mutation in familial and non-familial CRC patients was studied. At the molecular level, the 1100delC mutation eliminates the kinase domain and activity of Chk2 [127]. To evaluate the importance of 1100delC in CRC, Kilpivaara *et al.,* studied the frequency of the 1100delC allele in 662 CRC patients, including 149 familial and 513 non-familial cases [129]. The frequency was 2.6% for the whole patient cohort, 1.3% for familial and 2.9% for non-familial, which is not significantly higher than the expected frequency in the population (1.9%). Moreover, they observed a low frequency of the loss of the wt allele (17.6%) in the germline mutation carrier tumors, and the loss of the mutant allele in one tumor. Their study suggested no significant association between germline *CHEK2* 1100delC and familial or sporadic CRC, but a low penetrance effect of CHEK2 in CRC pathogenesis could not be ruled out. Moreover, another study by Meijers-Heijboer *et al.,* reported that the frequency of the 1100delC mutation was somewhat higher in MMR gene mutation carriers and in patients without MMR gene mutations compared with the controls. In addition, they reported that *CHEK2* 1100delC mutation was absent in 95 familial adenomatous polyposis families [130]. de Jong *et al.,* studied the mutation in 629 unselected CRCs, 230 controls and 105 selected CRCs diagnosed before age 50. In this study, the unselected CRC patients showed no increased frequency of the *CHEK2* 1100delC genotype compared to controls [131]. However, after stratifying unselected CRC patients according to defined genetic risk, a significantly increased frequency was observed [131]. There was no association of the 1100delC genotype with tumor localization, gender or age at diagnosis [131]. van Puijenbroek *et al.,* studied 564 familial colorectal tumors. They found that only a small percentage of patients presenting loss of *CHEK2* expression have the *CHEK2* 1100delC mutation [132]. The authors also concluded that Chk2 protein abrogation is not caused by the *CHEK2* germline variants R117G, R137Q, R145W, I157T and R180H in familial CRC [132]. Schutte *et al.,* reported a relatively high frequency of the 1100delC variant in families predisposed to combined breast and colon cancer [133]. In 2011, a meta-analysis of CHEK2 1100delC variant, found an association of CHEK2 1100delC variant with unselected CRC and familial CRC [134]. In conclusion, *CHEK2* mutations probably contribute, albeit with low penetrance, to CRC susceptibility. Moreover, it is noteworthy that not only Chk2 but other proteins implicated in G1/S are affected in CRC: p21 waf1/cip1 protein, which is a p53-inducible inhibitor of the cyclin-dependent kinases, and cyclins D and E which are key regulators of G1 progression. Abnormal p21 mRNA and protein levels have been reported in CRC [135–137]. Overexpression of cyclin D1 may be a marker of aggressive biological behaviour in CRC, and has been correlated with advanced stage [135]. Tort *et al.,* studied the alteration of several components along the p16-cyclin D1pRb-E2F-cyclin E pathway in human colorectal adenomas, concluding that cyclin D1 and cyclin E showed distinct clinical variables and biological behavior, with potential implications for individualized management of tumors with elevated cyclin D1 versus cyclin E [138]. Furthermore, p53 aberrations are among the commonest genetic changes in the development of CRC [139].

Therefore, several alterations of the checkpoints can be present in CRC, and may have an impact on its prognosis and possibly on the design of targeted treatments.

Targeting the Checkpoints—The common occurrence of checkpoint defects in cancer cells provides the rationale for targeting checkpoint pathways for therapeutic intervention [123]. When cancer cells harbor some checkpoint defects, they exhibit a greater dependence on the remaining checkpoint processes. Unlike normal cells with a full arsenal of checkpoints responses, tumors with defects in some checkpoint(s) could be deprived of their remaining checkpoint pathways(s), and consequently driven into death due to accumulation of excessive DNA damage [123]; a potential strategy could be targeting p53-deficient cancers that lack the G1 checkpoint and inhibiting the checkpoints kinases known to participate in the G2 checkpoint, such as ATM/ATR or Chk1 [123]. Consequently, checkpoint inhibition is an opportunity to selectively increase the effect of DNA damaging agents, when the checkpoint kinase is the kinase that remains intact.

Different chemical categories of checkpoint inhibitors have been identified: aminopyrazoles, indazoles, tricyclic compounds, ureas, carbamates, diazepinones, pyrimidines, benzimidazole quinolones, macrocyclic compounds, etc. [140–142]. Checkpoint inhibitors modulate the cellular response to DNA damage, leading to checkpoint abrogation, inhibition of DNA repair and changes in the regulation of cell death.

UCN-01 (7-hydroxy-staurosporine) targets Chk1, but also potently inhibits a number of other kinases including Chk2 and a number of the cyclin-dependent kinases. It is currently believed that the potentiation of DNA-damaging agents is primarily driven by the inhibition of Chk1 and, hence, abrogation of cell cycle arrest [143, 144]. In CRC cells, the complete growth arrest induced by WMC26 (bisimidazoacridonesin, a potential bisintercalating anticancer drug with CRC selectivity *in vitro* [145]) sensitizes to apoptotic death induced by UCN-01 [146]. This suggests that combining WMC26 and Chk1 inhibition may be an attractive treatment method for CRC, that exploits the highly tumor-selective activity of WMC26 [146]. Furuta *et al.,* showed that UCN-01 inactivates p21, allowing the repair of replication-mediated DSBs induced by Top1 [147]. This study provided the rationale for using camptothecins in cells with functional defects in the p21 pathway, and for combining camptothecins and cell cycle checkpoint inhibitors, such as UCN-01, in cancer chemotherapy [147]. The results by Petersen *et al.,* suggest that p53-dependent G1 arrest in both first and second cell cycles may protect human cancer cells from cell death after treatment with ionizing radiation (IR) and Chk1 inhibitors. Hence the idea to combine Chk1 inhibitors with IR to selectively target p53-deficient cancer cells becomes strongly attractive [148]. Xiao *et al.,* showed that Chk1 downregulation sensitizes tumor cells to the toxicity of paclitaxel in cell proliferation assays; Chk1 inhibition facilitates paclitaxel-induced M-phase entry by activation of Cdc2 kinase and accumulation of cyclin B1, the required cofactor for Cdc2 kinase activity [149]. Therefore, in addition to its role as an enforcer of S and G2checkpoints in response to genotoxic stress, Chk1 also plays a protective role in mitotic checkpoint to lessen mitotic catastrophe, and thereby limits cell death. Moreover, Xiao *et al.,* demonstrated that 5-FU activates Chk1 and induces an early S-phase arrest, through Chk1-mediated CDC25A proteolysis leading to inhibition of CDK2 [150]. Chk1 elimination stabilizes CDC25A protein product and results in the abrogation of the S checkpoint and resumption of DNA synthesis, which in turn leads to excessive accumulation of DSBs [150]. As a result, downregulation of Chk1 potentiates 5-FU efficacy through the induction of

premature chromosomal condensation followed by apoptosis [150]. As a consequence, Chk1 inhibition not only potentiates the toxicity of conventional DNA-damaging agents such as IR and topoisomerase inhibitors, but also enhances the toxicity of anti-microtubule agents [149] and antimetabolites in cancer cell lines [150]. All these studies suggest that inhibiting the G2 checkpoint is a “key element” to sensitize p53-deficient CRC cells to DNA damaging agents, anti-microtubule agents or antimetabolites.

Concerning Chk2, Castedo *et al.,* showed that inhibition of Chk2 facilitates the induction of mitotic catastrophe in HCT116 cells, thus sensitizing proliferating cells to chemotherapy-induced apoptosis [151]. Chk2 deficiency is associated with defective S-phase checkpoint, prolonged G2 arrest, and hypersensitivity to CPT [152]. Therefore there is a potential value for testing Chk2 status in colorectal tumors treated with Top1 inhibitor clinically. Theoretically, the combination of Chk2 inhibitors with oxaliplatin may further sensitize CRC cells to oxaliplatin treatment. Paradoxically, these inhibitors produced an antagonistic effect on the response to oxaliplatin, which was reversed by the reintroduction of Chk2 [153]. These observations may have important implications for the design of trials foreseeing the use of oxaliplatin in CRC therapy combined with Chk2 inhibitors [153]. At comparable concentrations in human colon cancer cells, cisplatin slowed down the replication phase and activated the G2/M checkpoint, whereas oxaliplatin activated the G1/S checkpoint and completely blocked the G2/M transition [154]. Chk2 inhibitors have been developed as sensitizers for chemotherapeutic agents. However, Chen *et al.,* reported that Chk2 activation alone led to potent inhibition of cancer cell proliferation; consequently, indirect activation of checkpoint kinases may be a novel approach for cancer therapy in selected genomic contexts, such as p53 wild-type CRCs [155]. Recently, we observed that in TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-treated colon carcinoma cells, Chk2 is activated and amplified the TRAIL-induced apoptosis by a feedback loop, activating the upstream caspase-8 [156]. From a therapeutic standpoint, our findings suggest that the level of Chk2 and activated P-Chk2-T68 in tumor tissues could have a prognostic value for predicting the efficiency of a TRAIL-based therapy. Moreover, our results provide a rationale for combining TRAIL with DNA-damaging agents. Indeed, DNA damage might sensitize tumor cells to TRAIL by preactivating Chk2 and the positive feedback loop that amplifies TRAIL-induced apoptosis. Such a mechanism may partly account for the known synergism between TRAIL and DNA damaging agents [157, 158]. These studies show the multifaceted nature of Chk2: indeed, Chk2 inhibitors could sensitize to chemotherapeutic agents, but in selected genomic context the indirect activation of Chk2 could itself be an approach for cancer therapy (for an in-depth analysis of the variability and the potential genomic determinants of Chk2 status *in vitro*, see also [128]).

Recently, some Chk inhibitors (for both Chk1 and Chk2) have shown their efficiency in enhancing DNA damaging agents in colon carcinoma cells [159, 160].

Inactivation of p53 increases the cytotoxicity of CPT in HCT116, which demonstrates a critical role of p53 as a G1 checkpoint regulator after CPT-induced DNA damage and suggests a rationale for the selectivity of CPT toward p53-deficient tumors [161]. Filder *et al.,* showed that PG490, triptolide, sensitizes tumor cells to Top1 inhibitors by blocking p53-mediated induction of p21 [162]. They also demonstrated that PG490–88, a derivative of

triptolide, causes tumor regression of CRC xenografts when administered alone, and synergizes with irinotecan (a camptothecin analog) to cause tumor regression [163]. In 2003, PG490–88 underwent a phase I clinical trial in patients affected by solid tumors [163]. PG490–88 caused tumor regression in some cases in combination with DNA-damaging agents, suggesting its role as an antineoplastic agent and chemosensitizer for the treatment of patients with solid tumors [163].

During the mitotic spindle checkpoint arrest, Sayed *et al.,* identified two novel roles for casein kinase 2 (*CK2*): a role in maintaining increased cyclinB/cdc2 kinase activity, as a component of G2 arrest; and a role in p53-mediated apoptosis [164]. Kaestner *et al.,* showed that treatment with MLN8054 (a selective Aurora Kinase A inhibitor) in human CRC cell lines induces defects in mitotic spindle assembly, which causes a transient spindle checkpoint-dependent mitotic arrest [165]. The cell cycle arrest is not maintained due to the ability of MLN8054 to override the spindle checkpoint. Subsequently, MLN8054-treated cells exit from mitosis and activate a p53-dependent postmitotic G1 checkpoint, which subsequently induces p21 and Bax, leading to G1 arrest followed by the induction of apoptosis. ZM447439, an Aurora Kinase B inhibitor, also interferes with normal chromosome alignment during mitosis and overrides the mitotic spindle checkpoint but allows a subsequent endoreduplication, although the compound potently activates the p53-dependent postmitotic G1 checkpoint [165].

In summary, the discovery and development of checkpoint inhibitors is an area of intense interest and active research. The preclinical data provide confidence that these inhibitors will be useful in clinical practice, mostly in combination with DNA-damaging therapies, for the improvement of CRC treatment.

PARP INHIBITORS IN CRC THERAPY: PRECLINICAL FINDINGS AND CLINICAL PERSPECTIVES

Poly(ADP-ribose) polymerases (PARP) are a family of 17 structurally related mammalian enzymes (reviewed in [166–169]). PARP1 is the most abundant member of the PARP family. It is normally associated with chromatin, and PARP1 activation is associated with transcription activation, in some cases in association with DNA cleavage by topoisomerase II beta (*TOP2B*) [170, 171]. PARP1 is strongly activated by DNA damage, including SSBs and DSBs. PARP catalyzes poly(ADP-ribose) (PAR) polymer formation transferring by nicotinamide adenine dinucleotide (NAD+). PARP polymerizes PAR to substrate proteins (histone, topoisomerase enzymes among others) including itself. The automodified PARP binds to DNA and recruits XRCC1, DNA ligase III (*LIG3*), DNA polymerase β (*POLB*) to the DNA damage sites and is thereby involved in base excision repair (BER) [172–174]. PARP, along with XRCC1, has also been implicated in the alternative non-homologous end joining (NHEJ) pathway of DSB repair [175, 176]. Finally, PARP inhibits the conversion of SSBs to DSBs [59]. Thus, PARPs have an important role in the maintenance of genomic integrity, making them promising targets for pharmacological strategy [177, 178]. PARP inhibitors are now used in an increasing number of clinical trials [179]. Because BRCA1 and BRCA2 are defective in many cancers and necessary for DSB repair, inhibition of PARP1 is

selectively effective in BRCA1/BRCA2-deficient cancer cells [180, 181]. Thus, PARP inhibitors provide a novel way to treat BRCA-deficient cells in combination with chemotherapy and radiation, while sparing normal cells.

PARP is closely related with CRC. In the early stage of colorectal carcinogenesis, *PARP1* mRNA overexpression was detected in 64 (70.3%) of the 91 tumors analyzed (65 adenomas and 26 submucosal (pT1) cancers). *PARP1* overexpression is significantly correlated with tumor size and histopathology [182]. In leucocytes of patients with familial adenomatous polyposis (FAP), who are genetically predisposed to colon cancer, stimulation of PARP activity upon radiation was absent comparing with that of healthy volunteers [183].

Single nucleotide polymorphisms (SNPs) in the *PARP1* gene could also modify the CRC risk. In an ancillary analysis of the Singapore Chinese Health Study, which 1,176 healthy control and 310 cancer patients (180 colon and 130 rectum cancers), a positive association between the *PARP1* codon 940 Lys/Arg and Arg/Arg genotypes and CRC risk was observed [184]. Polymorphisms in Val742 (Val742Ala) modifies the association of high red-meat content diets with risk of CRC [185]. These findings suggest that PARP1 might play an important role in tumorigenesis of CRC.

PARP inhibitors potentiate the cytotoxicity of several DNA damaging agents including ionizing radiation, alkylating agents and Top1 inhibitors (reviewed in [167, 178]). We recently published the results of the combination of veliparib with Top1 inhibitors in human cancer cell models [59]. Veliparib (ABT-888) synergized with CPT and induced DSBs. Moreover, siRNA knockdown of *XPF* or *ERCC1* enhanced the antiproliferative effects of veliparib in camptothecin-treated cells, suggesting the involvement of XPF-*ERCC1* in Top1-induced DNA damage as an alternative pathway from *PARP* and *TDP1*, and providing the rationale for combining inhibition of *PARP1* and Top1 in cancer with XPF-ERCC1 deficiency.

The tricyclic benzimidazole AG14361, a potent PARP inhibitor enhanced the radiosensitivity of CRC xenografts [186]. AG14361 (5 mg/kg) was intra-peritoneally administered daily for 5 days to CD-1 nude mice bearing LoVo or SW620 (CRC cell lines) xenografts, followed by 2 Gy irradiation. Under such treatment schedule, tumor growth was delayed by 37 days compared with a delay of 19 days (with local tumor irradiation alone) ($P = 0.008$). Veliparib potentiated radiation (2 Gy/d x 10) in a HCT116 CRC xenografted model, with a median survival time of 36 days compared with 23 days ($P < 0.036$, log-rank test) from radiation alone [187].

Beyond radiosensitization, PARP inhibitors also synergize with chemotherapy, such as temozolomide (TMZ) and irinotecan in colorectal tumor models. ABT-888 facilitates the conversion of TMZ-induced SSBs to DSBs and potentiates the cytotoxic effect of TMZ in the HCT116 cell line [188]. Oral administration of GPI-15427 enhances the antitumor efficacy of TMZ and irinotecan combination in HT-29 and LoVo xenografts [189]. Also GPI 15427 showed a protective effect on irinotecan-induced severe intestinal injury of jejunum and delayed diarrhea [189]. GPI-15427 did not exacerbate myelotoxicity induced by full dose TMZ.

Based on these preclinical data, four PARP inhibitors, olaparib (AZD2281, Astra-Zeneca, Inc.), veliparib (ABT888, Abbott, Inc.), iniparib (SSAR240550-BSI-201, SanofiAventis, Inc.) and CEP-9722 (Cephalon Inc.) have entered the clinical phase of experimentation in CRC tratment. According to the information from http://clinicaltrials.gov, several clinical trials are undergoing (last accessed January 20th, 2012). In a phase II trial, olaparib is used alone to treat previously-treated patients with stage IV, measurable CRC, stratified by microsatellite instability status, whereas a phase I trial assessing the safety/efficacy of olaparib/irinotecan combination in locally advanced or metastatic CRC has recently been completed. A phase I trial of veliparib in combination with irinotecan, 5-FU, Folinic Acid (FOLFIRI) to treat patients with advanced solid tumors including CRC was started in February 2010, and another phase I study, which evaluates the association of veliparib with CapeOX (capecitabine and oxaliplatin) in advanced solid tumors is ongoing. Also, a phase II study of veliparib in combination with TMZ in patients with heavily pretreated, metastatic CRC has been ongoing in Georgetown University Medical Center since October 2009 and will be completed in December 2012. Moreover, the phase I study of evaluating the safety and pharmacokinetics of iniparib and the study of CEP-9722 in combination with gemcitabine and cisplatin have also been initiated in patients with advanced solid tumors. Results from these studies will help to clarify the real potential of PARP inhibition as a strategy to sensitize CRC to current DNA damaging agents on clinic.

INTERACTIONS BETWEEN THE EGFR AND DDR PATHWAYS

The epidermal growth factor receptor (EGFR) has emerged as a relevant molecular target for cancer. The activation of EGFR results in activation of a series of downstream signaling pathways that ultimately regulate key cellular processes such as proliferation, survival, adhesion, migration and differentiation [see the review by Ballestrero A. *et al.,* in the present issue of CCDT and [190] (Fig. 3)]. EGFR is frequently dysregulated in CRC, and the overexpression of the receptor confers a poor prognosis [190–193]. Thus, EGFR has become an attractive target in the treatment of CRC. Buisine *et al.,* investigated the polymorphic simple sequence repeat in *EGFR* intron 1 (CASSR I) in CRC [194]. They detected mutations in CA-SSR I in 86 % of MSI-H (microsatellite instability with high frequency) CRCs and in 0 % of MSS (microsatellite stable) CRCs [194]. This indicates that *EGFR* gene could be a novel putative specific target of MMR-deficient CRCs [194]. Of interest, no association could be observed between *EGFR* expression and CA-SSR I in tumor or normal tissues [194]. Thus, CA-SSR I sequence does not contribute to the regulation of *EGFR* transcription in colon, and should not be considered as a promising predictive marker for response to EGFR inhibitors in patients with CRC [194]. Several strategies to inhibit the EGFR and its downstream signaling pathways are available in preclinical and clinical studies. They include monoclonal antibodies against the extracellular domain of the receptor and small-molecule inhibitors of its tyrosine kinase activity (see Ballestrero A. *et al.,* in the present issue of CCDT, [190], and ClinicalTrials.gov website). Erlotinib, a selective EGFR tyrosine kinase inhibitor, reduces thymidylate synthase (TS) activity in EGFR-expressing cells, probably due to cell cycle arrest in the G1 phase [195]. Combining erlotinib with pyrimidine trifluorothymidine (TFT) shuts down even more TS, possibly due to cell cycle interference [195]; consequently combining EGFR blockade with fluoropyrimidines has a molecular

rationale in CRC. Hiro *et al.,* showed that 5-FU-induced activation of EGFR followed by radiation in chemo/radioresistant SW480 cells results in up-regulation of ERCC1 [196]. On the contrary, 5-FU-induced degradation of EGFR followed by radiation in radiosensitive CRC cell lines resulted in down-regulation of ERCC1 [196]. These data suggest that there is a complementary interaction between EGFR and ERCC1, and that 5-FU-induced EGFR activation confers protection against radiation through activation of NER pathway [196]. Among the proteins activated by EGFR is the transcription factor STAT3. Vigneron *et al.,* observed that the EGFR-STAT3 oncogenic pathway up-regulates the EME1 endonuclease, reducing DNA damage after Top1 inhibition [197]. These results suggest the rationale for combining anti-EGFR therapy with Top1 inhibitors to sensitize drug-resistant colorectal tumors [197]. Gefitinib (Iressa®, Astra-Zeneca, Inc. and Teva, Inc.), a selective EGFR kinase inhibitor enhances the antitumor effect of oxaliplatin in CRC cell lines [198]. Gefitinib not only enhances cellular Pt-DNA adducts but also markedly inhibits their removal, which prolongs oxaliplatin pro-apoptotic effects [198]. Recently, the combination of a dimeric Affibody molecule (ZEGFR:1907)2 targeting EGFR with radiation has shown a decrease in the survival of HCT116 cells [199].

EGFR inhibition seems a logical therapeutic strategy to enhance CRC treatment with DNA damaging agents, and clinical and translational studies need to be pursued to completely understand the possible relationship between EGFR inhibition and DDR pathway modulation.

CONCLUDING REMARKS

In the present review, we have tried to depict the relationship between members of two essential cellular pathways, DDR and checkpoint, with CRC. We have explored several known and potential determinants that could be of importance in better targeting this disease, and we have illustrated the rationale lying at the foundation of two currently used DNA damaging agents, irinotecan and oxaliplatin. Clearly, our picture is only a blurred image of the magnificient fresco that makes up such complicated and wondrously intertwined pathways as DDR and checkpoints are. These last years have seen tremendous improvements in our understanding of DDR and checkpoints, as well as great and positive changes in CRC treatment, but a much greater effort needs to be undertaken to unveil the determinants of CRC resistance to chemoterapeutical agents, to optimize treatment strategies, and to identify new targets in the management of this disease.

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ABBREVIATIONS

APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3 homolog

BER	base excision repair
CIN	chromosomal instability
CRC	colorectal camcer
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
DDR	DNA damage response
DSB	DNA double-strand break
EGFR	epidermal growth factor receptor
ERCC1	excision repair cross complementation group 1
FAP	familial adenomatous polyposis
FHA	forkhead homology-associated
5-FU	5-fluorouracil
MMR	mismatch repair
MSI-H	microsatellite instability with high frequency
MSS	microsatellite stable
MVA	mosaic variegated aneuploidy
NAD	nicotinamide adenine dinucleotide
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PAR	poly(ADP-ribose)
PARP	poly(ADP-ribose) polymerase
SNP	single nucleotide polymorphism
SSB	single-strand break
TFT	trifluorothymidine
TMZ	temozolomide
Top1	topoisomerse
TRAIL	tumor necrosis factor-related inducing ligand apoptosis-
TS	thymidylate synthase

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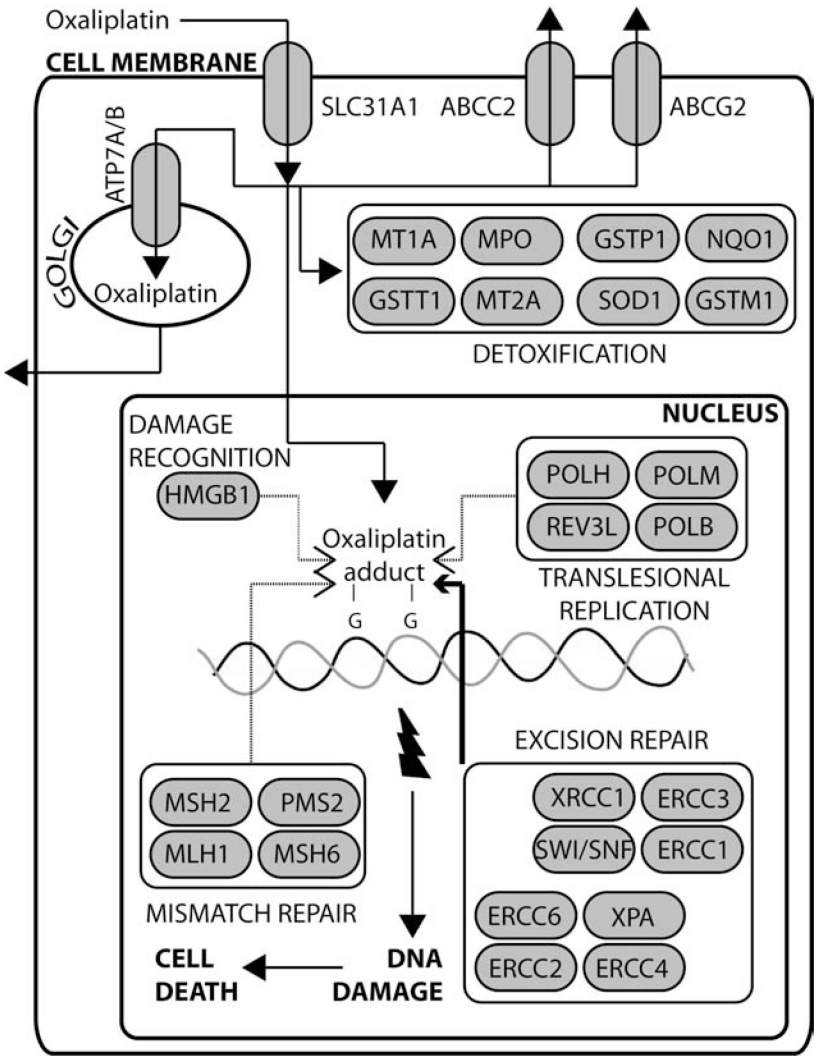

Fig. (1).

Oxaliplatin pathways: thin dotted arrows indicate pathways that are not deemed relevant in oxaliplatin DDR compared with cisplatin (this diagram has been reproduced with modifications from http://www.pharmgkb.org. Permission has been given by PharmGKB and Stanford University; copyright PharmGKB).

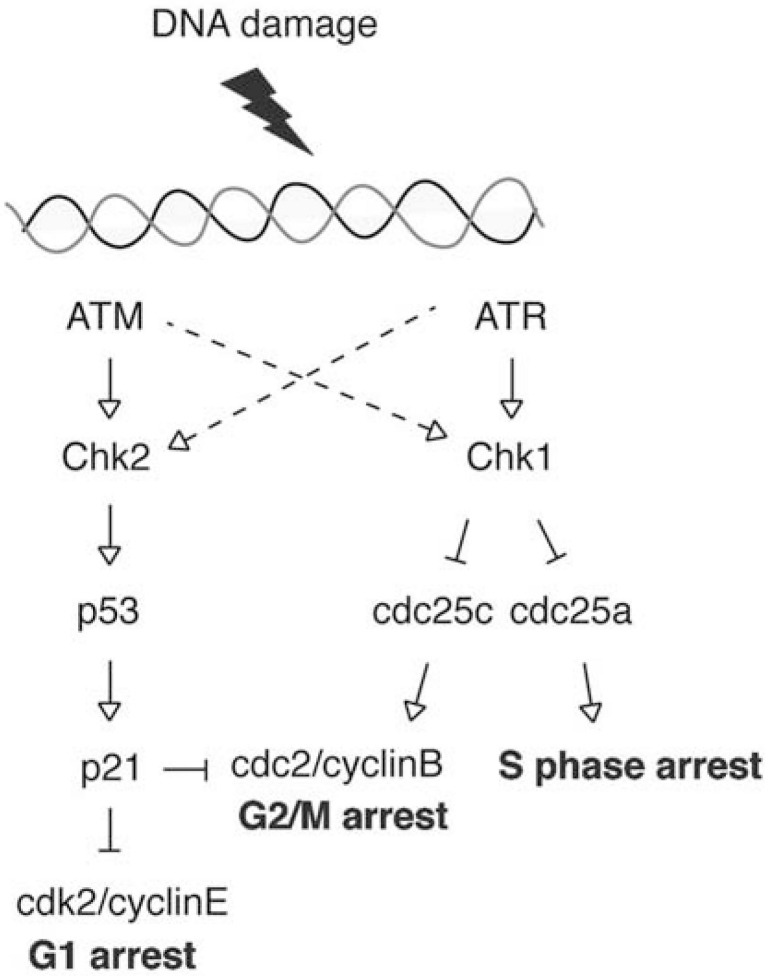

Fig. (2).
Cell cycle checkpoints.

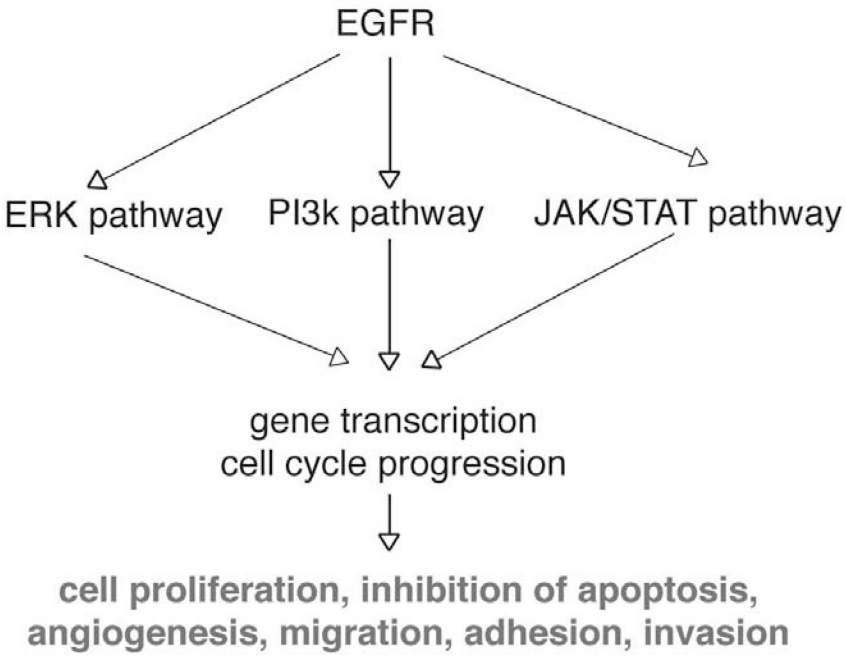

Fig. (3).
EGFR pathways.

Table 1.

Germline mutations in colorectal familial syndromes.

Syndrome	Involved Gene(s)	Affected Process	Actively Affecting DNA Integrity ?	References
Familial adenomatous polyposis (FAP)	APC	APC/β-catenin/Wnt pathway	Yes, chromosomal instability, aneuploidy	[13, 14, 20]
MYH-associated polyposis (MAP)	MYH	Base excision repair	Yes, chromosomal instability, aneuploidy	[13, 21–23]
Human non-polyposis colon cancer (HNPCC)	MSH2, MLH1, PMS1, PMS2, MSH6, TGFBR2, MLH3	Mismatch repair	Yes, microsatellite instability, increased mutation rate	[15, 24, 25]
Oligodontia-colorectal cancer syndrome	AXIN2	APC/β-catenin/Wnt pathway	Not described	[14, 20, 26]
Familial Finnish colorectal cancer predisposition	CHEK2	Cell cycle checkpoint	Yes, DNA integrity maintenance	[27, 28]
Peutz - Jeghers syndrome	STK11	Wnt pathway, cell cycle	Not described	[29–33]