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DNA polymerases in the risk and prognosis of colorectal and pancreatic cancers

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Abstract

Human cancers arise from the alteration of genes involved in important pathways that mainly affect cell growth and proliferation. DNA replication and DNA-damages recognition and repair are among these pathways and DNA-polymerases that take part in these processes are often involved in cancer onset and progression. Based on their main functions we can recognize replicative and reparative DNA-polymerases. These participate in different steps of cancer development. In fact, replicative polymerases can affect cancer onset, while reparative polymerases are mostly involved in the acquisition of drug resistance. Several patients affected by colorectal cancer harbour germline or somatic mutations within the proofreading domain of replicative polymerases delta (Polδ) or epsilon (Polε). Inherited germline mutations within the genes encoding for these two polymerases (*POLD1* and *POLE*) represent a genetic risk factor for the development of CRC. Somatic alterations of the same genes, on the other hand, can drive the carcinogenesis by leading to the accumulation of many other mutations throughout the genome. While replicative polymerases can lead to cancer development when damaging mutations alter their functions, reparative polymerases are often involved in the acquisition of drug resistance for their physiological role. In fact, their ability to repair and bypass DNA-damages counteracts the effect of most anticancer drugs. In addition, however, the alteration of the expression level of these polymerases that sometimes characterize different type of cancers can exacerbates this aspect. For example, all of the DNA-polymerases involved a damage bypass mechanism, known as translesion synthesis (TLS), with the only exception of polymerase theta, are downregulated in CRC. Conversely, in pancreatic ductal adenocarcinoma (PDAC), most of these polymerase result upregulated. This suggests that different types of cancer can rely on different reparative polymerases to acquire drug resistance. Here we will examine all of the aspects that link DNA-polymerases with CRC and PDAC.

Introduction

DNA-polymerases are enzymes involved in cellular processes that range from the accurate duplication of genomic and mitochondrial DNA to the repair and tolerance of DNA-damage. In addition to the fundamental functions they have in physiological conditions, polymerases are involved in many processes that can affect the onset, development, and prognosis of human cancers, including the response to therapies. Replicative DNA-polymerases delta (Polδ) and epsilon (Polε), for example, are the most accurate among human polymerases and are responsible for the faithful replication of genomic DNA. The high fidelity of these polymerases is a feature that heavily depends on their proofreading activity conferred by the 3'-exonuclease domain. Over the years, however, a number of studies have led to the identification and characterization of many different alterations within the 3'-exonuclease domain of these polymerases. Some of them consist in germline alterations conferring increased risk of cancer occurrence. Others are somatic mutations that resulted as possible drivers of carcinogenesis (1). The extremely high fidelity of replicative DNA-polymerases is not enough by itself to assure the faithful replication and the stability of genomic DNA. Indeed, replicative DNA-polymerases are not able to deal with the wide range of DNA damages constantly caused by endogenous and exogenous sources. A large variety of low fidelity polymerases, the reparative DNA-polymerases, evolved to guarantee genome replication and stability even in the presence of DNA lesions. The study of these polymerases led to the progressive characterization of their biological functions and, at the same time, highlighted also their involvement in cancer development and progression. In fact, every time a new polymerase has been identified and characterized (along with its physiological role), some kind of cancer-related function was also reported. Polymerase beta (Polβ), encoded by *POLB* gene, is the main polymerase involved in the base excision repair (BER). Its activity is essential for DNA replication and maintenance but some variants of *POLB* have been associated with an increased risk of developing ovarian, bladder and breast cancer (2–4). Moreover, somatic mutations of *POLB* confer a "mutator phenotype" and are considered strong drivers of carcinogenesis (5). Others DNA polymerases involved in DNA repair possess much less fidelity, such as those belonging to the Y-family. These polymerases are specialized in bypassing DNA-damages

during replication through a mechanism known as "translesion synthesis" (TLS). Their ability to promote DNA replication through several types of lesions prevents the collapse of stalling replicative forks and help to maintain the stability of the genome at the cost of increased mutation rates. However, this ability to bypass DNA-damages can also confer resistance to some anticancer drugs. For example, polymerase eta (Polη), one of the main polymerases involved in TLS, can confer resistance to platinum-based drugs (e.g. cisplatin, oxaliplatin and carboplatin) as well as to replicative stress-inducing agents, including AraC and gemcitabine (6). Other examples are polymerase theta (Polθ) that seems to confer resistance to replicative stress-inducing agents AraC and hydroxyurea (7) and polymerase nu (Polν) whose inhibition sensitizes cells to cross-linking agents (8). Given their large involvement in all these mechanisms, it is not surprising that DNA-polymerases are emerging as risk factors, prognostic biomarkers and possible therapeutic targets in cancer. In this review, we will explore the functions of both replicative and reparative DNA-polymerases, in relation to colorectal cancer (CRC) and to pancreatic ductal adenocarcinoma (PDAC). We will illustrate also how the features of these polymerases could be exploited in order to increase the efficiency of the existing chemotherapeutic drugs.

B-family polymerases are potential risk factors, prognostic markers, and therapeutic targets in CRC

The faithful replication of genomic DNA in eukaryotes is mainly dependent on the activity of three B-family DNA-polymerases, i.e. polymerase alpha (Polα), polymerase delta (Polδ), and polymerase epsilon (Polε) (9), the only multimeric human polymerases. Among them, Polα, a four subunits polymerase/primase complex, encoded by *POLA1, POLA2, PRIM1,* and *PRIM2* genes (10), is specialized in the assembly of RNA-DNA primers, while two high fidelity polymerases, Polδ and Polε, participate in the genome replication by catalysing the synthesis of lagging and leading strands (11). Polδ and Polε are multimeric proteins encoded by *POLD1, POLD2, POLD3, POLD4* and *POLE, POLE2, POLE3, POLE4* genes respectively. The presence of a 3'-exonuclease domain makes Polδ and Polε the most accurate human polymerases, and confers them a central role in maintaining genome stability and accuracy (12). In addition to their functions in DNA replication, Polδ and Polε are also

involved in several DNA damage repair and bypass mechanisms (13,14). The importance of these polymerases in a fundamental cellular process, such as DNA replication, led to generate the hypothesis of a strong involvement in human cancers, hypothesis confirmed by several studies.

Polα is a target of a new class of anticancer drugs and a promising predictive marker in CRC

One of the many issues related to the treatment of patients affected by CRC, is the absence of biomarkers predicting the response to a preoperative chemoradiation (CRT) in local advanced rectal cancer (LARC). Based on a study carried out by Palme et al. (15) on 10 responder and 16 non-responder patients, *POLA1,* that encodes for the catalytic subunit of Polα, showed promising features as such a type of biomarker. Indeed, the expression level of *POLA1*, combined with that of 3 other genes (*Gng4*, *c-Myc* and *Rrm1*), allowed to discriminate between responders and non-responders with a sensitivity of 60% and a specificity of 100%. Although these results are quite interesting as, for the first time, they show that Polα could be employed as predictive marker for response to CRT in LARC, their clinical relevance still need to be carefully evaluated. Another aspect that is worth to be mentioned is the role that Polα could play in the development of a new class of anticancer drugs, the retinoid related molecules (RRMs). RRMs are synthetic derivatives of retinoic acid that exert an apoptotic activity on a wide range of tumor cells, as well as in animal model (16–19). Two of these RRMs (CD437 and ST1926) have been specifically studied in CRC cell lines and xenograft models with the aim to verify their cytotoxic effect and to understand the molecular mechanisms through which they exert this effect. In a study carried out by Han et al. (20) on 6 CD437-resistant clones and 13 CD437-sensitive clones derived from HCT116 cell lines, *POLA1* was identified by whole-genome sequencing as the only gene to harbour at least one missense mutation in each of the CD437-resistant clones. Six different mutations were identified (C691Y, L700S, L764S, I768T, A772D, and A772T) and functional experiments showed that the introduction of the allele L764S into HCT116 and HeLa cells (using CRISPR-Cas9 technique) conferred resistance to CD437. The idea that Polα could have a direct role in determining the sensitivity to CD437 was confirmed further by experiments showing that CD437 caused an inhibition of Polα activity. Moreover, the concentration of CD437 required to inhibit Polα in CD437-resistant clones was at least 10-fold higher than the concentration needed in CD437-

sensitive clones. Furthermore, this inhibition was shown to be the result of a direct interaction between CD437 and the catalytic subunit of Polα. A similar study was conducted by Abdel-Samad et al. (21) on another compound that belongs to the class of RRMs: the adamantyl retinoid ST1926. At sub-μM concentrations, ST1926 induced apoptosis and cell cycle arrest in CRC cells HT29, HCT116 and LoVo, but not in their normal-like counterpart (NCM460). Moreover, it slowed down tumor growth in xenograft mice. Similarly to what happened with CD437, HCT116 clones that were resistant to ST1926, showed missense mutations within *POLA1* gene and ST1926 was able to abrogate *in vitro* primer extension by Polα. Together, these studies confirmed the antitumor properties of CD437 and ST1926 in CRC and suggested that a direct interaction between Polα and these two RRMs is responsible for these properties. Another interesting aspect is the relation between the primase subunit encoded by *PRIM1* gene, and some inhibitors of ATR. Inhibitors of ATR, such as VX970 and VE-822, showed promising results *in vitro, in vivo* and in clinical trials as combined or stand-alone anticancer drugs. In particular, V-822 showed a strong synergistic effect with oxaliplatin in different CRC cell lines and in animal models and restored sensitivity in oxaliplatin resistant cells (22). According to a screening carried out by Hocke et al. (23) on 288 DNA-repair genes, six of these genes, including *PRIM1* and *POLD1,* showed a synthetic lethality with ATR/CDK1 meaning that the inhibition of one of these genes had detrimental effects only on cells with impaired ATR/CDK1 (and vice versa). A subsequent study conducted by the same group to confirm and further characterize the relation between *PRIM1* and ATR inhibition, showed that siRNA-mediated depletion of *PRIM1* increased the sensitivity to ATR inhibitors (AZD6738 or VE-822) in a panel of CRC cell lines (DLD-1, SW480 and RKO) (24). Since *PRIM1* is a component of the Polα/primase complex, it is not excluded that the inhibition of any other component of this complex can synergize with an impairment of ATR, thus leading to an enhanced anticancer effect of Polα inhibitors mentioned above (CD437/ST1926), in ATR-deficient cells.

Replicative polymerases Polδ and Polε are associated with an increased risk of colorectal cancer

In addition to the already mentioned relationship between *POLD1* and *ATR,* one of the most relevant role that the other two replicative B-family DNA-polymerases, Polδ and Polε, play in CRC is related

to the impairment of their proofreading activity. This was ascribed to rare variants within *POLD1* and *POLE* genes encoding their 3'-exonuclease domain. Germline alterations of *POLD1* and *POLE* were linked for the first time to an increased risk to develop CRC, by two studies conducted by Tomlinson's group. Using a combination of whole-genome sequencing and Sanger sequencing they identified two germline variants within the 3'-exonuclease domain of *POLD1* (S478N) and *POLE* (L424V) that were associated with susceptibility to CRC. *In vitro* experiments carried out on yeast also suggested a functional pathogenic role for these variants, as both of them caused a substantially increased mutation rate. The predisposing condition determined by alterations of *POLD1* and *POLE* was defined by the authors as "polymerase proofreading-associated polyposis syndrome" (PPAP) (25,26). Since then, numbers of studies focused on the identification of *POLE* and *POLD1* germinal mutations among unexplained familiar, early-onset CRC and polyposis. These studies confirmed the pathogenic role of *POLE* L424V (27–31) and *POLD1* S478N (30,31) mutations and led to the identification of novel putative pathogenic variants associated with CRC risk, therefore contributing to the characterization of PPAP, as summarized by Buchanad et al. (32). These considerations, along with the results of a study conducted to assess the relative and cumulative risks of CRC for subjects that carry germline mutations within *POLD1* and *POLE* genes, strongly recommend the screening for polymerase proofreading mutations, especially in unexplained familial cases. Moreover, the increased risks of developing CRC associated with *POLE/POLD1* pathogenic variants encourage to consider annual colonoscopy screening and clinical management guidelines for all carriers of *POLE/POLD1* pathogenic mutations, similar to those recommended for people with Lynch syndrome (32). Conversely, a study conducted on PDAC indicated that *POLE* hotspot mutations are quite rare in PDAC and that testing *POLE* may be useful under certain circumstances (e.g. in very young patients or in members of cancer prone families) but not on a routine basis (33).

Polζ is an unusual member of B-family polymerases and it is involved in CRC predisposition and drug resistance

Polymerase zeta (Polζ) is a multimeric DNA-polymerase encoded by *REV3L* and *REV7* genes. It is an unusual member of B-family polymerases as, in contrast with Polδ and Polε, is a low fidelity enzyme

that lacks the 3'-5' proofreading exonuclease activity. Polζ mainly acts as an extender polymerase during TLS, a mechanisms addressed further on this review. As we will see for other polymerases involved in the tolerance of DNA-damages, the ability to bypass different types of DNA lesions can have opposite consequences. Indeed, polymerases involved in TLS act as tumor suppressors by promoting genomic stability but also confer resistance to chemotherapeutic drugs. Accordingly, downregulation of *REV3L* expression and alterations within *REV3L* coding sequence have been associated with an increased risk to develop different types of cancer, included CRC. On the other hand, several *in vitro* and *in vivo* experiments showed that the inhibition of Polζ can sensitize human cancer cells to platinum-based chemotherapeutic agents, suggesting that Polζ may be involved in chemotherapy acquired resistance (34). In a study conducted by Brondello et al. on 74 subjects affected by colorectal carcinoma a significant downregulation of *REV3L* has been reported in tumor tissues compared with adjacent normal tissues, with 54.1% of the tumors that displayed more than twofold downregulation of *REV3L,* suggesting that Polζ may play an important role in the progression of colon cancer (35). The idea of an involvement of Polζ in CRC tumorigenesis seems to be supported also by other studies that led to the identification of rare and common variants of *REV3L* associated with risk or CRC. In a recent study, whole genome sequencing performed on germline-tumor matched DNA samples from 16 unrelated Spanish patients affected by CRC led to the identification of germline variants within some DNA repair genes (included *REV3L* R187W) that may be involved in CRC predisposition(36). Even if functional studies and replication in additional cohorts are needed to confirm the actual role of these variants, other evidence exist that link Polζ to CRC risk. A study carried out on a Czech cohort, composed by 1832 CRC patients and 659 controls, showed that the rare variant AA genotype of *RELV3L* rs3204953 G>A was associated with a significant increased risk of CRC (AA vs. GG; OR 2.32; 95% CI 1.27–4.25; p = 0.006; and AA vs. GG+GA; OR 2.28; 95% CI 1.24–4.17; p = 0.008) (37). Another study carried out on two different Chinese cohorts composed by 516 CRC cases and 503 controls and 421 CRC cases and 446 controls led to the identification of another *REV3L* SNP that was significantly associated with CRC risk. Indeed, in the first cohort TT and CT genotypes of rs462779 C>T were associated with a 1.80 and 1.43 fold increased risk of CRC respectively and similar results were obtained with the second replication cohort (38). While these

data suggest that an impairment of Polζ functions can be considered a risk factor for CRC, evidence also exist that Polζ can confer drug resistance in different types of cancer. Indeed, siRNA mediated inhibition of *REV3L* led to increased sensitivity to cisplatin in lung (39) and glioma (40) cancer cells. It is also interesting to notice that the inhibition of Polζ increased the cytotoxic effect of oxaliplatin in cervical cancer and lymphoma cells (41) and also increased esophageal cancer cells sensitivity to 5-fluorouracil (5-FU) (42). Since a combination of 5-FU and oxaliplatin is widely used as adjuvant chemotherapy in CRC, these latter findings should encourage the evaluation of Polζ as a possible therapeutic target in order to overcome drug resistance in CRC.

X-family polymerases are involved in BER and NHEJ and can affect carcinogenesis and therapy response in CRC

The three polymerases that belong to the X-family, Polβ, Polλ and Polμ, encoded respectively by *POLB, POLL* and *POLM* genes, are low fidelity polymerases that lack a proofreading domain and function mainly in DNA repair pathways. While the role of Polμ seems to be mainly restricted to V(D)J recombination during lymphocytes maturation, Polβ and Polλ participate, through their gap-filling activity, to different DNA-repair pathways such as the base excision repair (BER) and the non-homologous end-joining (NHEJ). Despite their functions partially overlap, Polβ mainly participates in BER and preferentially fills small gaps, while Polλ exert its gap filling activity mainly during double-strand breaks (DSBs) repair mediated by NHEJ. However, *in vitro* evidences show also that Polλ can substitute Polβ activity in BER, suggesting a possible backup role under certain circumstances. Polβ and Polλ seem to take part also in some specialized form of TLS. Indeed, both of them are able to bypass and repair of 7,8-dihydro-8oxoguanine (8oxoG) and abasic sites lesions *in vitro*. Since BER repairs at least 20.000 DNA lesions per day per cell and given the importance of Polβ in this pathway, it is quite obvious that alterations of Polβ activity can result in detrimental effects for the cell. Similarly, it is not surprising that the participation of Polβ and Polλ in important repair pathways also results in their involvement in drug resistance mechanisms.

POLB somatic variants confer a mutator phenotype and lead to carcinogenesis in CRC

Polβ is the main DNA-polymerase implicated in base excision repair (BER) through its gap filling activity. Alterations of Polβ functions can result in unfilled gaps that serve as substrate for recombination and result in genomic instability. Data from several small-scale studies showed that about 30% of human cancers express Polβ variants and suggested that an impairment of Polβ function can increase the rate of mutagenesis and lead to carcinogenesis, as reviewed by Starcevic et al. (5). For years, the observation that cancer cells harboured a number of mutations that was incredibly higher than expected suggested that somatic alterations in one or more genes involved in the maintenance of the genomic integrity could be responsible. Thus, many investigators started to analyse the somatic alterations in genes known to participate in DNA replication/repair, in order to identify those genes that, if altered, were able to confer what so called a "mutator phenotype". One of the first studies to explore the hypothesis that sporadic mutations of *POLB* could confer a mutator phenotype in CRC was published in 1992 by Wang et al. (43). Since then, numbers of other works focused on the identification of potentially pathogenetic somatic alterations within the catalytic domain of Polβ in CRC. Some of these led to the individualization and characterization of an 87-bp deletion, corresponding to the deletion of 29 aminoacidic residues comprised between position 208 and 236, in subjects affected by CRC. As shown by several *in vitro* experiments, this deletion lead to the production of a truncated protein (PolβΔ) that is able to inhibit the gap filling activity of the wild type (WT) Polβ resulting in the acquisition of a mutator phenotype (44,45). Bhattacharyya et al. showed that PolβΔ causes a dominant inhibition of WT Polβ gap filling activity, probably through an interaction with X-ray cross complementing group 1 (XRCC1), further supporting the pathogenetic significance of this variant (46). An *in vivo* study that examined the potential tumorigenic activity of PolβΔ in nude and transgenic mouse models, confirmed once more the hypothesis that PolβΔ could be a potent driver of carcinogenesis in CRC and other kind of cancers (47). These works on PolβΔ paved the way for other studies that led to the identification of several somatic mutations of *POLB* in CRC samples. For some of these alterations, *in vitro* experiments suggested a pathogenetic role. In fact, S229L, E295K, G231D and K289M induced cellular transformation probably trough a reduced gap filling activity that led to increased chromosomal aberrations (48–51) and E288K caused an increase in the frequency of mutations at AT base pairs in vitro, leading to genomic instability (52,53). Overall,

these data reveal a scenario in which *POLB* plays an important role in the acquisition of the "mutator phenotype" that drives tumorigenesis in CRC leading to the accumulation of a huge number of mutations within each cancer cell. In addition, two SNPs of unknown functional significance, A165G and T2133C, which lie respectively within intron 2 and 10 of *POLB*, have been strongly associated with increased overall survival in PDAC patients. In particular, the 2 homozygous variant genotypes were associated with a doubling of survival time compared with the wild-type and heterozygous variant genotypes (54). Nonetheless, additional functional studies are required in order to elucidate the actual role of these SNPs.

Inhibition of Polβ and Polλ sensitized CRC cell lines and xenografts to chemotherapeutic agents

As already mentioned, the tolerance to DNA damage conferred by reparative DNA-polymerases, can be a double edge sword as it allows the maintenance of genome integrity, thus preventing carcinogenesis, but can also counteract the genotoxic effects of many anticancer drugs, conferring drug resistance to cancer cells. In fact, some of the drugs used in cancer therapy, such as temozolomide (TMZ) and cisplatin, induce DNA-damages that BER can recognize and repair. Thus, given the role that Polβ and Polλ play in this repair pathway, their inhibition could be an attractive strategy to increase the responsiveness to these drugs. In agreement with this hypothesis, Iwatsuki et al. (55) reported a correlation between the expression levels of Polβ and a poorer prognosis in CRC patients and showed that the inhibition of Polβ by short interfering RNA (siRNA) sensitizes CRC cells CaR-1 to cisplatin. A group headed by Narayan, carried out a series of studies in order to identify small molecules to employ as specific inhibitors of Polβ and to assess whether these inhibitors could enhance the therapeutic efficacy of TMZ in CRC. These studies led to the identification of two molecules, NSC-666715 and NSC-124854 that were able to inhibit Polβ functions by preventing its interaction with APC (56–58). By *in vitro* and *in vivo* experiments, the authors showed that the inhibition of Pol β led to an increase cytotoxicity of TMZ probably by inducing S-phase cell cycle arrest also resulting in senescence/apoptosis activation. Another work conducted by Strittmatter et al. (59) led to the identification of a series of highly potent Polβ and Polλ inhibitors based on the rhodanine scaffold. The authors identified 10 small molecules that selectively inhibited Polλ and 14

that selectively inhibited Polβ and were more potent compared with other already known inhibitors. Moreover, two of these inhibitors sensitized CRC Caco-2 cells to TMZ. Even though these results are probably still far from reaching a clinical significance, they can be considered a prove of principle that the inhibition of BER mechanism through Pol β targeting could be a viable strategy to increase the sensitivity to some anticancer drugs that may be employed in the treatment of CRC.

A-family polymerases can participate in the bypass of DNA cross-links

The polymerases that belong to the A-family became an attractive subject of study after the identification and characterization of *Mus308* in Drosophila. This gene was quite unusual as it encoded for both, an N-terminal helicase domain and a C-terminal polymerase domain. Moreover, *Mus308* mutations increased the sensitivity to DNA cross-linking agents and this led to the hypothesis that, in Drosophila, Mus308 could be involved in DNA interstrand repair (60). The idea that a family of specialized DNA-polymerases could participate in DNA cross-links repair was quite attractive. In fact, since many platinum compounds currently employed as anticancer drugs act as cross-linking agents, human polymerases involved in bypass or repair of DNA lesions caused by cross-linking agents, could potentially confer cancer cells resistance to these drugs. Thus, the identification and characterization of human genes involved in DNA cross-link repair could have been an important step for the development of novel anticancer drugs. This idea driven the works that led to the identification of two human genes related do Drosophila *Mus308*: *POLN* and *POLQ* (61,62).

Pol ν is involved in ICL repair in human cells and seems required for an efficient HR

In 2003 Marini et al. identified a human gene, *POLN* (encoding for polymerase nu; Polν), which shared a high similarity with the C-terminal polymerase region of *Mus308* (61). The homology between Mus308 and Polν suggested a possible involvement of Polν in DNA cross-link repair and prompted for deepened its role in cancer. Even if the *in vitro* activity of Polν seemed to be limited to the bypass of 5S-thymine glycol lesions (63), studies in human cell lines told a slightly different story. In fact, siRNA mediated knock down of Pol ν, resulted in an increased sensitivity to cross-linking agents such as cisplatin and mitomycin in HeLa cells, suggesting an involvement of Pol ν in ICL

repair (8). To date, no data are available regarding a specific involvement of Polν in CRC. However, given the evidences about its role in ICL repair, further studies may be worth.

Pol θ protects cancer cells against DSB and replicative stress and can affect chemotherapy efficiency in CRC.

Like its Drosophila ortholog Mus308, human polymerase Polθ (encoded by *POLQ*) has a unique structure as it contains a helicase domain at the N-terminus and a polymerase domain at the C-terminus and, similar to what happened with Polν, many research groups tried to elucidate its possible involvement in human cancer, especially its implication in drug resistance. Regarding the involvement of Polθ in CRC, many studies are concordant in reporting an overexpression of *POLQ* in the majority of CRC cases and a strong association between this overexpression and a poor prognosis (64,65). Even if, to date, there are no direct evidences that Polθ can affect therapy response in CRC patients, a recent work showed an association between a common missense variant of *POLQ* (rs3218651 A>G) and prognosis of CRC patients treated with a combination of 5-FU and oxaliplatin (37). Two excellent reviews by Yousefzadeh MJ and Wood RD highlight many of the relevant features of Polθ as purified enzyme and in a cellular context (66). In *in vitro* experiments, purified Polθ showed the ability to incorporate an A residue opposite to abasic (AP) site and continuing the extension from the incorporated nucleotide (67). Moreover, Polθ was able to extend mismatched strand (68) and to bypass thymine glycols, even though with a lower efficiency compared with Polν (63). Furthermore, experiments conducted in a cellular context and *in vivo* strongly suggested an involvement of Polθ in microhomology-mediated end joining (MMEJ), a key cellular process used as an alternative way to repair DSB, especially in HDR-deficient cells (66). Accordingly, Polθ was able to promote the survival of HR-deficient cancer cells and its inhibition sensitized these cells to DSB inducing agents (69,70). Despite these evidences, some observations indicate that the role of *POLQ* is probably not limited to its involvement in MMEJ in HR-deficient cancer cells. Indeed, Goullet de Rugy et al. (7) reported that the expression of *POLQ* in patients affected by breast cancer (n=221), lung cancer (n=94) and CRC (n=52) did not correlate with that of other genes that encode for MMEJ factors. Vice versa, the authors found a positive correlation between the expression of *POLQ* and that of some HDR genes

involved in the management of stalled replication fork under replicative stress, a condition defined as the "perturbation of error-free DNA replication and slow-down of DNA synthesis" that is typically found in cancer cells (71). In addition, a siRNA-mediated depletion of *POLQ* sensitized RKO cells to replicative stress inducing agents Ara-C and hydroxyurea (7). Thus, Polθ seems to contribute to those mechanisms that confer tolerance to replicative stress in cancer cells. Many endogenous and exogenous factors can induce replicative stress. Two of the most used chemotherapeutic drugs in CRC, along with their main effects, also induce replicative stress: 5-FU, by reducing the size of the available dNTPs pools, and oxaliplatin, by inducing the formation of DNA cross-links. The ability to induce replicative stress is an important feature of anticancer drugs as the accumulation of stalled replicative forks can results in their collapse, inducing a mitotic catastrophe and eventually leading to cell death (71). These considerations, along with the observation that SNPs within *POLQ* coding sequence are associated with therapy response in CRC patients (37), suggest that Polθ may affect the response to chemotherapy in CRC probably through its involvement in the mechanisms that cancer cells use to manage the replicative stress induced by some anticancer drugs. Potentially, *POLQ* could be an intriguing therapeutic target as its inhibition could sensitized cells to chemotherapeutic drugs such as (but not limited to) 5-FU and Oxaliplatin.

Y-family polymerases are mainly involved in DNA damage bypass by TLS

As mentioned above, many anticancer drugs cause replication stress. Every type of factor that somehow interferes with DNA replication can cause a slowdown in the replication and even a stall of replicative fork. If not bypassed, the lesion can result in the collapse of the fork and eventually lead to cell death or chromosomal breakages with important rearrangements of the karyotype. Thus, the ability to bypass DNA damages during replication is a vital function for cancer cells as it confers tolerance to replicative stress and can ultimately result in chemotherapy resistance. The TLS is the main pathway through which cells bypass DNA damage during replication. We already mentioned the involvement of several polymerases in TLS (e.g. Polζ and Polθ) and briefly discussed their implication on the acquisition of drug resistance in cancer cells but an entire class of DNA polymerases exists which members are specifically specialized in TLS: the Y-family. The members of the Y-family are

four monomeric polymerases, Polη, Polκ, Polι and Rev1 encoded respectively by *POLH*, *POLK*, *POLI*, and *REV1* genes and consist in an N-terminal domain that contains the catalytic site and a C-terminal regulatory domain that is involved in protein-protein interactions. As for all of the other polymerases encountered so far, *in vitro* studies led to the characterization of the enzymatic properties of each of these polymerases. It is important to notice though, that these *in vitro* studies analyzed the activity of the purified enzyme and are not always representative of the real functions that a given polymerase exerts in its natural cellular context. Nonetheless, these studies can provide important notions to understand the possible role of these polymerases *in vivo.* While it is beyond our scope to analyze every single *in vitro* function of these polymerases, some of their properties can help to understand their role in TLS and are worth to be considered. Polη seems to be specialized in the bypass of UV-induced lesions, in particular of cyclobutane pyrimidine dimers (CPDs) and, accordingly, a depletion of Polη confers an extreme sensitivity to UV radiations and predispose to skin cancer. The fact that alterations within *POLH* coding sequence are associated with the development of xeroderma pigmentosum (XPG), a genetic disease characterized by an elevated photosensitivity that predispose to the development of skin cancer, further confirms the fundamental role of Polη in the tolerance of DNA damages caused by UV radiations (72,73). Polη can also incorporate nucleotides opposite to thymine-thymine 6-4 photoproduct (74) and, similar to the other members of the Y-family, is able to incorporate nucleotides opposite to an abasic site. On the contrary, Polκ is unable to incorporate nucleotides opposite to these kind of lesions (except for the abasic sites) and seems more specialized in handling bulky lesions generated by polycyclic aromatic hydrocarbons (PAHs). In addition, both, Polη and Polκ, seem to have an important role in the tolerance of oncogene-induced replication stress in cancer cells (75,76). The role of the other two members of the Y-family is more elusive. The functions of Polι seem to overlap with that of Polη. The ability of Polι to bypass the same lesions as Polη but with a lesser efficiency, led to the hypothesis that Polι, under certain circumstances, may act almost as a backup for Polη (77,78). Rev1, the last member of this family, seems to have mainly a "structural" function, as it represent a scaffold that, by interacting with the other polymerases, allows their recruiting within the stalled replicative fork. Three excellent reviews cover almost all of the aspect related to these polymerases (79–81). In the effort to simplify as much as

possible, we could image TLS as a process that involves polymerases with three main functions. Rev1 has a structural function, regulates the access of the other polymerases to the damaged site and mediates the polymerase switch. Polη, Polκ and Polι incorporate nucleotides opposite to lesion, with Polη and Polκ that are specific for different types of lesion and Polι that functions as a backup for Polη. Lastly, Polζ extends the nascent strand past the lesion **(Figure1)**. It is important to notice, though, that the real scenario is much more complex, with many other proteins involved in the TLS and with the functions of polymerases themselves that are partially overlapping.

Figure1

Polη expression associates with sensitivity to chemotherapy in vivo and confers resistance to platinum compounds in vitro

As discussed, the involvement of Y-family polymerases in TLS makes them possible factors involved in the development of drug resistance in many human cancers. From this point of view, the most characterized polymerase among this family is Polη. The expression levels of Polη associates with chemotherapy response in a wide range of malignancies, including non-small cell lung cancer (NSCLC) (82), squamous cell carcinomas of head and neck (HNSCC) (83) and gastric adenocarcinoma (84). The role of Polη in conferring resistance to chemotherapy is confirmed also by *in vitro* studies showing that a depletion of Polη leads to a sensitization to some anticancer drugs in different cell lines (85–88). Since there are no studies that assessed the role of Y-family polymerases and CRC, we decided to focus on those that analyzed the relationship between Polη and the effectiveness of some compounds that are widely used as adjuvant chemotherapy in CRC. In a small-scale study that involved 80 metastatic gastric adenocarcinoma patients treated with FOLFOX or XELOX, Teng et al. analyzed the relationship between Polη expression and clinical outcome in patients as well as sensitivity to oxaliplatin in 4 different gastric cancer cell lines. The authors reported that the cell lines with a lower level of expression of Polη showed a greater sensitivity to oxaliplatin and that an overexpression of Polη was significantly associated with shorter survival in patients treated with FOLFOX or XELOX (84). While this study only highlighted an association between Polη expression and response to FOLFOX/oxaliplatin and could not be considered a direct evidence of the involvement of Polη in drug resistance, other works confirmed this hypothesis by *in vitro* experiments.

Albertella et al. showed that xeroderma pigmentosum-variant (XP-V) cell lines, characterized by impaired Polη functions, were dramatically more sensitive to oxaliplatin than the same cells complemented with functional Polη (89). Cruet-Hennequart et al. obtained similar results comparing the sensitivity to oxaliplatin of XP30RO cells (harboring deletion of 13-bp within the second exon of *POLH*) and TR30-9 (XP30RO containing a wild type *POLH* transgene), with XP30RO being significantly more sensitive (90). In addition, structural analysis studies showed the physical interaction between Polη and platinum compounds-DNA adducts, providing a molecular basis for understanding how Polη can tolerate DNA-damage caused by these drugs and further confirming its role in bypassing platinum-DNA adducts (91–93). Even if these studies were not conducted on CRC patients/cell lines, FOLFOX, a combination of folic acid, 5-FU and oxaliplatin is currently the standard regimen for adjuvant chemotherapy in CRC. Thus, the association between Polη expression and FOLFOX response in gastric cancer patients, along with the higher sensitivity to oxaliplatin in Polη deficient cell lines, strongly suggest the opportunity to explore further the role of Polη in CRC as well.

Conclusions

Maintaining genome integrity and DNA fidelity through every single cell cycle, is not an easy task for the cell. Many different proteins are require to provide the cell with the ability to faithfully duplicate its genetic pool and when alterations in these proteins occur and progressively accumulate, they can impair this ability and eventually result in cell death or, sometimes, in cancer onset. DNA-polymerases belong to this class of proteins whose functions are extremely important for cell survival and proliferation and have been extensively studied for their involvement in many types of human cancers. Here we showed that, somehow, almost every DNA-polymerase could be link to CRC. We retrieved from TCGA (https://portal.gdc.cancer.gov) some information about missense, non-sense and frameshift mutations affecting polymerases genes in CRC and PDAC. As shown in **Figure2A**, alterations within *POLs* genes were quite frequent in CRC (TCGA-COAD) but much less in PDAC (TCGA-PAAD). Moreover, replicative polymerases Polδ (*POLD1*) and Polε (*POLE*), were mutated in 9% of the subjects from TCGA-COAD cohort and resulted the most frequently mutated polymerases

along with Polζ (*REV3L*) and Polθ (*POLQ*). These observations are in accordance with the crucial role of these polymerases. In fact, Polδ and Polε that are directly involved in DNA replication, and Polβ, which contributes to the accuracy of replication trough BER, have a huge impact in the onset and development of CRC. As shown, many studies linked germline mutations of *POLD1* and *POLE* to an increased risk to develop CRC. Consistently with their functions, there are also evidences that somatic alterations within the proofreading domain of Polδ and Polε, by leading to the accumulation of a massive number of mutations, can act as potent drivers of tumorigenesis (94). When we further analyzed the data from TCGA-COAD, we found that the number of simple somatic mutations (SSM) was significantly higher among CRC patients that harboured at least one alteration within *POLD1, POLE* or *POLB* genes, as shown in **Figure2B**. This seems to confirm the idea that, under certain circumstances, Polδ, Polε and Polβ could became potent drivers of carcinogenesis and lead to development of hypermutated tumors. While replicative polymerases can take part in tumorigenesis and increase the risk of developing CRC, reparative DNA-polymerases seem to affect mainly the prognosis and drug response. Despite many *in vitro* experiments showed that TLS polymerases can provide the cells with an increased resistance to anticancer drugs, Pillaire et al. (65) reported a generalized downregulation of reparative DNA-polymerases, with the only exception of *POLQ*. To confirm this observation, we performed an expression analysis on TCGA-COAD cohort using GEPIA 2 (http://gepia2.cancer-pku.cn). The results were quite consistent with the data reported by Pillaire et al. except for the upregulation of *POLB* **(TableI)**. Nonetheless, when we carried out a survival analysis we found an association between *REV1* expression and prognosis in microsatellite stable (MSS) CRC, and between *REV3L* and a survival in PDAC **(Figure3A and 3B)**, suggesting that some TLS polymerases may affect the prognosis, even in CRC patients, in spite of their generalized downregulation. On the other hand, the same survival analysis did not shown any correlation between POLQ expression neither in CRC nor in PDAC. Since Goulley de Rugy et al. (7) suggested that Polθ might have a role in tolerance of replicative stress by preventing the collapse of stalled replication forks, we analyzed the correlation between the expression of *POLQ* and the expression of a gene signature composed by 5 genes involved in the protection of stalled replicative forks (*RAD51, RUVBL1, BLM, BRCA1, BRCA2*). Even if we could not manage to found any correlation between

POLQ expression and the overall survival, the coexpression of *POLQ* with this 5 genes signature **(Figure3C)**, along with the results of the studies that we reported in this review, strongly suggest that Polθ is an important component of the mechanism responsible for the management of stalled DNA replication forks. Thus, along with the other proteins that take part in this mechanism, Polθ could confers tolerance against sustained replicative stress, and probably decrease cell sensitivity to some anticancer drugs.

Figure3

Acknowledgements

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Legends

Fig.1: schematic representation of translesion synthesis by Y-family polymerases and the extender polymerase Polζ. When replicative polymerases (Polδ or Polε) encounter a DNA lesion, replication stops and TLS polymerases are recruited to the lesion site **(A)**. Y-family polymerases Polη or Polk (depending on the type of lesion) insert a nucleotide opposite lesion **(B)**, and Polζ completes the bypass of the lesion by extending the DNA filament for some bases away from the lesion site **(C)**. After a short extension of the filament by Polζ, TLS complex dissociates and replicative polymerases can restart the replication **(D)**.

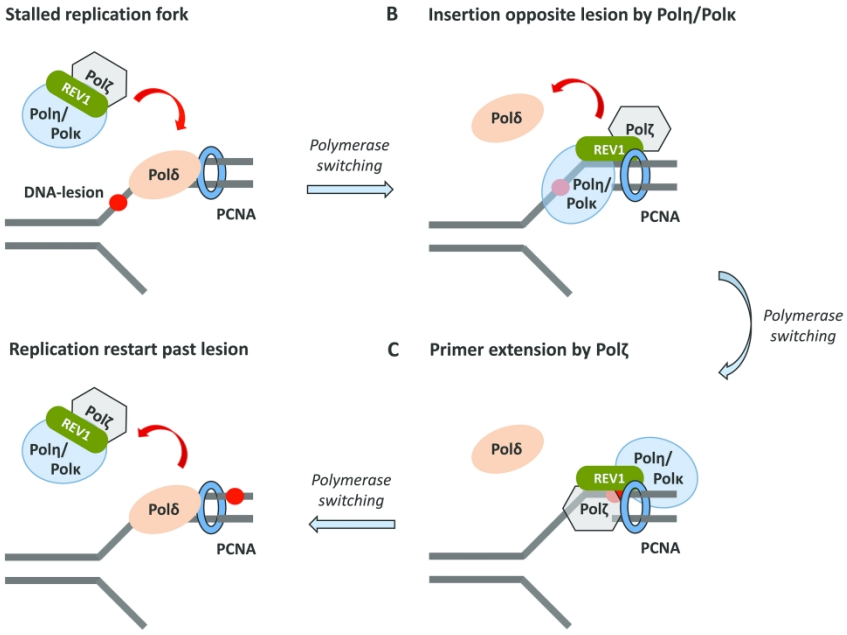
Fig.2: percentage of colorectal cancer (CRC) or pancreatic ductal adenocarcinoma (PDAC) samples with at least one missense, nonsense or frameshift mutation within a polymerase encoding gene according to TCGA using TCGA-COAD cohort for CRC and TCGA-PAAD cohort for PDAC **(A)**. Comparison between the number of simple somatic mutations found in the genome (21.098 sequenced genes) of CRC patients with mutated (n=43) or wild type (n=409) *POLB, POLD1* or *POLE,* according to TCGA (TCGA-COAD cohort) **(B)**.

Fig.3: survival analysis performed using GEPIA 2 and referring to TCGA datasets. Comparison between the overall survival of CRC patients (TCGA-COAD) expressing high or low level of *REV1* mRNA **(A)**; the same analysis carried out on PDAC patients (TCGA-PAAD) expressing high or low level of *REV3L* mRNA **(B)**. Correlation analysis to assess the co-expression of *POLQ* with a 5 genes signature (*RAD51, RUVBL1, BLM, BRCA1, BRCA2*) involved in the protection of stalled replication forks. The graph shows the p-value and the Pearson correlation coefficient (R) **(C)**.

Table I: relative expression of polymerases encoding genes on colorectal cancer (TCGA-COAD) and pancreatic ductal adenocarcinoma (TCGA-PAAD) compared with normal tissues. Data obtained from GEPIA 2 using TCGA and GTEX as datasets.

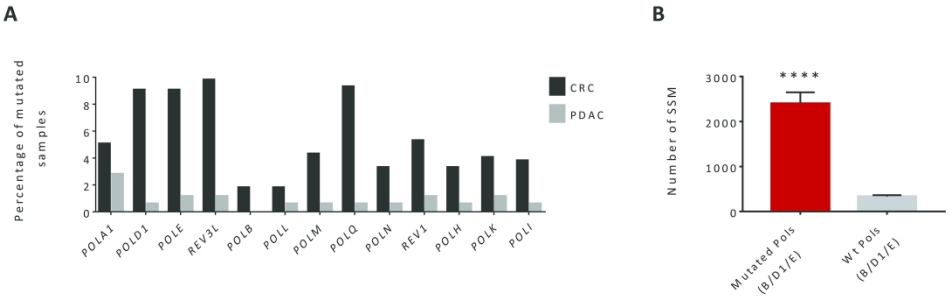
Gene	Protein	Role	Expression in TCGA-COAD	Expression in TCGA-PAAD
POLA1	Polα	Replication	up-regulated	up-regulated
POLD1	Polδ	Replication	up-regulated	up-regulated
POLE	Polε	Replication	unchanged	unchanged
REV3L	Polζ	TLS	down-regulated	unchanged
REV1	REV1	TLS	down-regulated	up-regulated
POLH	Polη	TLS	unchanged	up-regulated
POLK	Polκ	TLS	down-regulated	up-regulated
POLI	Polι	TLS	down-regulated	down-regulated
POLQ	Polθ	MMEJ/TLS/HDR	up-regulated	up-regulated
POLN	Polν	End processing	down-regulated	unchanged
POLB	Polβ	BER	up-regulated	up-regulated
POLM	Polμ	BER/NHEJ	unchanged	unchanged
POLL	Polλ	NHEJ	unchanged	unchanged

Figure1



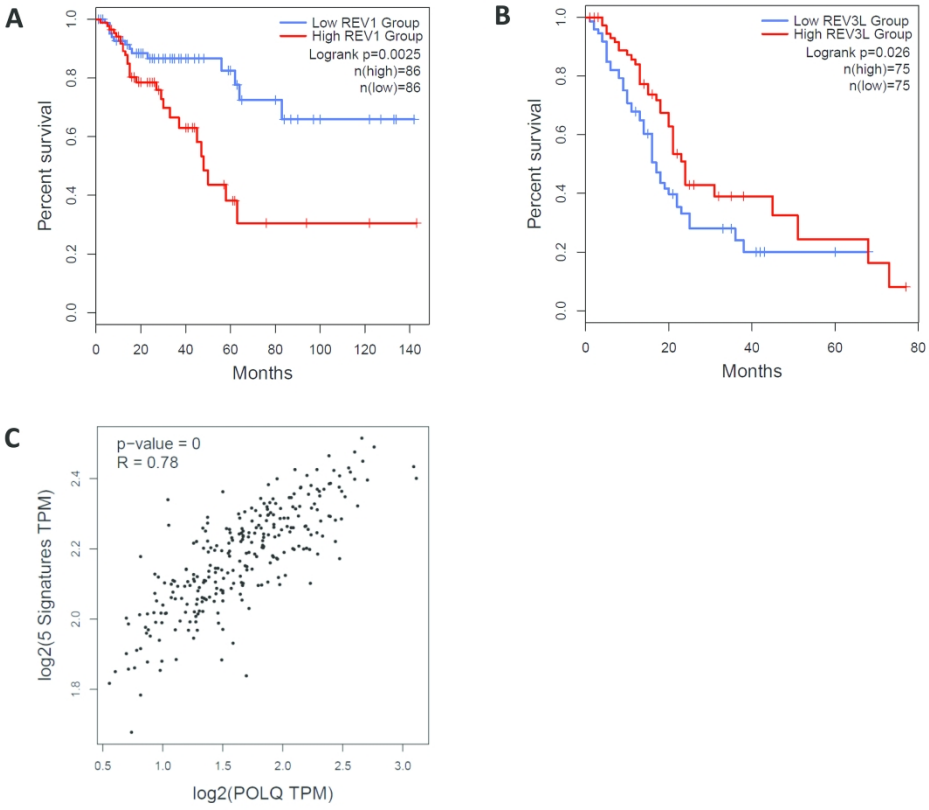
164x104mm (600 x 600 DPI)

Figure2



184x66mm (600 x 600 DPI)

Figure3



143x123mm (600 x 600 DPI)