Published in final edited form as:

Mutat Res. 2009 November 2; 670(1-2): 79–87. doi:10.1016/j.mrfmmm.2009.07.007.

Characterization of DNA polymerase β splicing variants in gastric cancer: the most frequent exon 2-deleted isoform is a non coding RNA

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Abstract

DNA repair polymerase β (Pol β) gene variants are frequently associated with tumor tissues. In this study a search for Pol β mutants and splice variants was conducted in matched normal and tumor gastric tissues and blood samples from healthy donors. No tumor associated mutations were found while a variety of alternative Pol β splicing variants were detected with high frequency in all the specimens analysed. Quantitative PCR of the Pol β variant lacking exon 2 (Ex2 Δ) and the isoforms with exon 11 skipping allowed to clarify that these variants are not tumor- neither tissue-specific and their levels vary greatly among different individuals. The most frequent Ex2 Δ variant was further characterized. We clearly demonstrated that this variant does not encode protein, as detected by both western blotting and immunofluorescence analysis of human AGS cells expressing HA tagged-Ex2 Δ . The lack of translation was confirmed by comparing the DNA gap-filling capacity and alkylation sensitivity of wild type and Pol β null murine fibroblasts expressing the human Ex2 Δ variant.

We showed that the Ex2 Δ transcript is polyadenylated and its half-life is significantly longer than that of the wild type mRNA as inferred by treating AGS cells with actinomycin D. Moreover, we found that it localizes to polyribosomes suggesting a role as post-transcriptional regulator. This study identifies a new type of DNA repair variants that do not give rise to functional proteins but to non coding RNAs that could either modulate target mRNAs or represent unproductive splicing events.

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Conflict of interest statement

The authors declare that there are no conflicts of interests.

1. INTRODUCTION

Base excision repair (BER) is the main enzymatic pathway for the repair of structurally non-distorting and non-bulky lesions that arise by either endogenous or exogenous sources [1]. Recent studies indicate that mutations or changes in the expression level of BER genes might lead to genomic instability [2,3]. DNA polymerase β (Pol β) (HGNC:9174) is the main BER DNA polymerase [4–6]. It is encoded by a single-copy gene that is expressed at low levels (~6 mRNA molecules per cell) throughout the cell cycle [7] and is inducible under stress [8–9]. The protein is folded into two distinct domains, each associated with a specific functional activity: the 8kDa amino-terminal with DNA binding and lyase activity and the 31kDa carboxy-terminal that is the catalytic domain. Because of its moderately high error-rate, Pol β is considered an error-prone DNA polymerase. Indeed, several studies have shown that overexpression of this polymerase leads to increased spontaneous mutation frequency [10], microsatellite instability [11], chromosome instability and tumorigenesis [12]. Overexpression of Pol β that is a frequent event in tumor tissues (approximately 30% in a large variety of cancers analysed [13]) can enhance resistance to chemotherapeutic agents [14]. A smaller percentage (12–20%) of tumours is characterised by Pol β underexpression [13]. Pol β haploinsufficiency may result in genomic instability as shown in young mice where half the gene dosage leads to increased mutagenic response to carcinogen exposure [15]. Many types of cancer present deletions of chromosome 8p, where the Pol β gene maps, in association with the more aggressive forms [16–17]. Complete loss of Pol β is lethal in mice and Pol β null embryo fibroblasts [5] are hypersensitive to killing, mutagenesis and chromosomal damage induced by alkylating agents and less efficient in DNA repair as compared to wild type cells [18]. Mutations in the Pol β gene have been found in colon, prostate and in gastric cancer tissues [19–23]. Functional analysis of three of these mutations (K289M, I260M and E295K) showed decreased fidelity in DNA synthesis and induction of genetic instability [24–25].

It is becoming increasingly clear that, besides gene mutations and alterations of gene expression, errors in epigenetic processes, such as methylation, transcription, RNA processing and translation may have deleterious consequences on genome stability. An epigenetic process which has a crucial role in maintaining the normal flow of genetic information is pre-mRNA splicing. In particular, alternative splicing is implicated in regulating the temporal and spatial expression of many genes, by selection of different splice sites [26]. However, splice variants are not only the products of a legitimate alternative splicing process but their occurrence has also been associated with human pathologies [27]. Multiple Pol β splice variants have been identified in many cell types, including cancer cells, and the exon 2-deleted isoform (Ex2 Δ) has been shown to be the most frequent variant [28–30]. However, little is known about the function of these splice variants and the mechanism by which they are produced (regulated or aberrant splicing?).

In this study a search for Pol β mutants and splice variants was conducted in gastric specimens from cancer and healthy individuals and in gastric cell lines. Neither somatic nor hereditary mutations were found while a variety of alternative splicing variants were detected in all samples analysed. The molecular characterization of the most common Ex2 Δ revealed that it is a non-coding RNA with feature compatible with a role as post-transcriptional regulator.

2. MATERIALS AND METHODS

Clinical samples and cell lines

Gastric tissues were collected from gastric cancer patients or healthy individuals subjected to surgery or gastroendoscopy respectively. All subjects signed an informed consent form, and provided a permission to tissue sample removal. Tumor and normal tissue samples were immediately snap frozen in liquid nitrogen and cryopreserved until use. All the gastric tumors

were confirmed histologically. This study was approved by the institutional review committee. Gastric cancer cell lines (AGS, NCI-N87 and SNU-16) were obtained from American Type Culture Collection and cultured according to the provider. SV40 transformed wild type and Pol β -deficient mouse embryonic fibroblasts were cultured as previously described [5].

DNA and RNA isolation

Cells/tissues were disrupted by using Mixer Mill 300 (Qiagen). The Qiagen Rneasy Fibrous Tissue kit was used for RNA extraction according to manufacturer's instructions. For DNA extraction, cells/tissues were homogenated in lysis buffer (10mM Tris-HCl pH 8.0, 10mM EDTA, 10 mM NaCl; 0.5% SDS) and DNA purified by using phenol/chloroform standard procedure.

Genomic DNA analysis

PCR—Amplification was performed for 30 cycles, by using primers (Invitrogen) and annealing temperatures shown in Table 1 (supplementary information).

Automatic sequencing—PCR products were purified and sequenced by ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with primers reported in Table 1 (supplementary information). Big Dye Terminator Cycle sequencing kit was used according to manufacturer's instructions (Applied Biosystems).

mRNA analysis

RT-PCR—Total RNA (1 μ g) was used as substrate for reverse transcription PCR performed by Invitrogen SuperScript One-step RT-PCR with platinum Taq kit or Invitrogen SuperScript III kit (for polyadenylated mRNA amplification), according to manufacturer's instructions. Amplification was performed for 35 cycles using standard conditions at the annealing temperature of 60°C with primers shown in Table 1 (supplementary information).

Nested-PCR—Amplification of 1 μ l of RT-PCR product was performed for 30 cycles using standard conditions at the annealing temperature of 60°C, with the following primers: 5' ATGAGCAAACGGAAGGCGCCGAGG 3'; 5' AAGCTGGGATGGGTCAGGAGAACATC 3'; 5'TCATCAGCGAATTGGGCTGAAATATTTG 3' and 5' CGCTCCGGTCCTTGGGTTCCCGG 3' [31]. The entire set of primers shown in ref. [31] were used to characterize Pol β splicing pattern.

cDNA sequencing—Pol β full length cDNA molecules were purified on agarose gel, cloned into pCR 2.1 vector (TA cloning kit, Invitrogen) and then sequenced as described above by using standard primers.

QPCR analysis—Total RNA (1 μ g) was retrotranscribed by using the High Capacity cDNA Archive kit (Applied Biosystems), according to the protocol provided by the manufacturer. mRNA isoforms that retain exon 2 (wild type Pol β mRNA) or lack exon 2 (Ex2 Δ Pol β mRNA) or exon 11 (Ex11 Δ Pol β mRNA) were quantified by using primers (900 nM) and MGB-probes (200 nM) (Applied Biosystems) shown in Table 2 (supplementary information). Inventoried TaqMan gene expression assays (Applied Biosystems) were used for the amplification of the endogenous controls (GAPDH, HGPRT or 18S). QPCR reactions were run in ABI PRISM 7000 SDS and analysed by ABI PRISM 7000 SDS software v1.1 with RQ Study v1.0.

mRNA stability—AGS cells were harvested at different times (0–8 hours) after addition of actinomycin D (1 μ g/ml f.c.) (CAS # 50–76-0, Sigma-Aldrich). RNA extraction and QPCR

were then performed as described above. Both GAPDH and HGPRT housekeeping genes were used as endogenous control in QPCR.

Polysome fractionation—A pellet of 8×10^6 AGS cells was lysed in 500 μ l of a buffer containing 10mM Tris-HCl at pH 7.4, 10mM NaCl, 10mM $MgCl_2$, 1% Triton-X100, 1% sodium deoxycholate, 1mM 1,4-bis-sulfanybutane-2,3-diol, 50 μ g/ml cicloheximide (CAS # 66–81-9, Sigma-Aldrich), 100 units/ml RNase inhibitors (Roche Diagnostic). The lysate was incubated for 5 minutes on ice and then centrifuged at 14000 rpm for 5 minutes at 4°C. The supernatant was layered onto a 5–70% linear sucrose gradient and centrifuged at 37000 rpm for 150 minutes at 4°C. The gradient was collected in 15 fractions from the top to the bottom and RNA extracted from each fraction by phenol/chlorophorm standard procedure.

Cloning and transfection

Wild type and Ex2 Δ Pol β cDNA molecules from AGS cells were cloned into pcDNA4/HisMax (Invitrogen) expression vector, by using one-step cloning strategy (“TOPO Cloning”, Invitrogen) and then transfected into wild type/Pol β null mouse fibroblasts or AGS cell line. Empty vectors were used as control. Transfection was performed by using lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol and 48 hours from transfection, zeocin (CAS # 181494-14-4, Invitrogen) was added (50, 250 and 125 μ g/ml for Pol β null and wild type fibroblasts and AGS cells, respectively). Wild type and Ex2 Δ Pol β cDNA were also cloned into a derivative of pcDNA 3.1 (Invitrogen), pcDNA-HA3, that was obtained by inserting three HA-tags in the multiple cloning sites. The cloning strategy allows the production of proteins with three HA-tags at the amino terminus. Pol β cDNAs and pcDNA-HA3 were digested with BamHI and XbaI restriction endonucleases (Biolabs) and then ligated by using standard procedure. At least two independent clones per cell type were tested in each assay.

When the proteasome inhibitor was used, 24 hours after transfection cells were incubated with MG132 (CAS #133407-82-6, Calbiochem) at a final concentration of 5 μ M for 7 hours at 37°C.

Western blotting

Whole cell extracts were prepared as described in [5] and probed with anti-Pol β polyclonal antibody [6] or anti-HA monoclonal antibody (clone 3F10).

Immunofluorescence

Cells were fixed with 2% formaldehyde in PBS for 10 minutes and permeabilised with 0.25% Triton-X100 in PBS for 10 minutes before being incubated with anti-HA monoclonal antibody for 1 hour. Goat anti-rat fluorescein-conjugated secondary antibody (Cappel) was applied for 30 minutes, followed by counterstaining with 0.5 μ g/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

Gap-filling assay

The gap-filling assay was performed as described in [6] by using oligonucleotides from MWG-Biotech AG. A time-dependent kinetics was carried out by incubating the DNA substrate in the presence of whole cell extracts and radiolabeled $\alpha^{32}P$ -dCTP for increasing periods of time at 37°C.

Cell growth rate

Cell growth rate assay was performed as described in [32]. Briefly, cells were seeded at a concentration of 40000 cells/well in 6-well plate, treated with methyl methanesulfonate (MMS,

CAS # 66-27-3, Sigma-Aldrich) (0.5–5 mM) and then counted 3 days after treatment. Results are expressed as the number of treated cells relative to untreated cells (% control growth).

3. RESULTS

Lack of gene mutations but frequent splicing events characterize Pol β in normal and tumor tissues from gastric cancer patients

Genomic DNA from 5 gastric tumours and 3 gastric cancer cell lines (AGS, NCI-N87 and SNU16) and cDNA from 14 gastric tumours were screened for Pol β somatic and/or germline mutations. In the case of genomic analysis, amplification and sequencing of exons 1 to 14, exon α and splicing junctions were performed. Pol β cDNAs were cloned into plasmids, amplified and then screened for Pol β full length molecules before sequencing. No mutations were detected either in the exon sequences or in the splicing junctions, suggesting that this type of event is infrequent in our gastric cancer (GC) patients.

The analysis of Pol β cDNAs by RT-PCR of RNA extracted from normal and tumour gastric tissues of 20 GC patients and 3 gastric cancer cell lines, by using primers designed to amplify the entire coding sequence, revealed the presence of two major bands on agarose gel: a product corresponding to full length Pol β cDNA (1013 bp) and a lower molecular weight product, corresponding to cDNA molecules that had lost one exon at least. Both normal and tumour tissues were characterized by these two Pol β cDNA forms (Figure 1A, lanes 2 and 3). Multiple bands characterized the RT-PCR products of the AGS gastric cells (lane 4) revealing the frequent occurrence of Pol β splicing products. RT-PCR products were then used as substrate in a second PCR (nested-PCR), which permitted us to define the Pol β splicing pattern. An example of the splicing profile of normal and tumor tissues derived from one patient with gastric carcinoma is provided in Figure 1B. As summarized in Table 3 a wide variety of Pol β splicing variants were observed in both normal and tumor tissues from all GC patients analysed, as well in the gastric cell lines. Exon 2 skipping was ubiquitous thus confirming that it is a predominant Pol β variant. Frequent loss was recorded for exon 5 and 6 in combination. A few cases of exon 9 loss also were observed. As expected from its low frequency, the Pol β isoform containing exon α insertion was not detected in any of the specimens. It is of note that a similar pattern of Pol β exon skipping was observed for normal and tumor tissue from the same subject, but a large inter-individual variability in the splice variant profile was present.

This nested PCR strategy did not allow us to detect those splice variants that originate from loss of exons that are template for PCR primers (e.g. exon 1, 3, 4, 7, 8, 10, 11, 13, 14). To obtain the complete spectrum of Pol β variants a pool of Pol β cDNA molecules from the AGS gastric cell line were cloned into a mammalian expression vector and sequenced. DNA sequencing of 35 Pol β cDNAs (Table 4) confirmed the unfrequent insertion of exon α (1/35) and the frequent loss of exon 2 (26/35), alone (18/35) or in combination with other exons (7/35). Exon 5, 6, 9 and 11 were also confirmed to be skipped but in combination with other exons. Exon 2 loss with retention of exon 3 occurred even in splice variants with multiple exon losses, as previously reported for bladder cancer Pol β splicing variants [29]. Interestingly, one variant characterized by the loss of exon 11 (Ex11 Δ) was identified. This variant has been previously described to act as dominant negative regulator of Pol β activity [33].

The expression levels of Pol β and exon 2- and 11 splicing isoforms are variable and not cancer-associated

Overexpression of Pol β has been described in a large variety of tumors [13]. We took advantage of our set of matched normal and tumor gastric tissues from the same patient to check whether there is indeed a link between the expression levels of Pol β splicing variants and cancer. The most frequent Ex2 Δ variant and the Ex11 Δ isoforms were selected together with the wild type

Pol β to run QPCR in paired normal and tumor tissues from 7 subjects. It is of note that we focused our analysis on retention or loss of exon 2/11; thus, what we consider as wild type isoforms are all transcripts which retain exon 2 (or 11) but may have lost other exons. The same approach was used for Ex2 Δ variant and Ex11 Δ isoforms quantification.

A large variation in the ratio between Pol β expression level in tumor versus normal tissues was detected indicating that overexpression of Pol β is not a unifying feature of GC (Figure 2A). Data from a larger set of samples confirmed this finding [34]. When the same analysis was extended to Ex2 Δ mRNA levels the profile was similar to that recorded with Pol β wild type (Figure 2B). The relative expression (tumor versus normal) of the Ex2 Δ splicing variant paralleled that of wild type suggesting a possible regulatory link between transcription of Pol β and Ex2 skipping. The level of the Pol β isoforms presenting the skipping of exon 11 also was measured (Figure 2C). The expression level of these isoforms also was variable but they were detectable in both normal and tumor tissues.

The question of the relative frequency of Ex2 Δ versus wild type mRNA in tissues from cancer patients and healthy subjects was also addressed. Ex2 Δ mRNAs were highly represented in both normal and tumor tissues at levels comparable with the wild type transcripts (Figure 2D). This isoform also is highly frequent in specimens from healthy individuals either gastric mucosa (N1 and N2) or blood samples (B1 to B4).

These findings confirm that exon 2 as well as exon 11 skipping are not cancer-related and show that Ex2 Δ variant occurs at high frequency in different tissues from healthy individuals.

Ex2 Δ Pol β variant mRNA is not translated

The loss of exon 2 with retention of exon 3 is predicted to produce a 26 amino acid product (~2.9 kDa) that would include the first 20 amino acids of the ssDNA binding dRP lyase domain of Pol β (Table 4). Small proteins (of the order of 2–4 kDa) are at the limit of detection of western blotting techniques but if the exon 2 skip splice variant evades the premature termination codon (PTC) by alternative translation initiation sites [30] larger truncated proteins are expected to be produced.

To characterize the translation product of exon 2 skipping, Pol β wild type (wt) and null cells were transfected with an expression vector containing human Ex2 Δ cDNA. Pol β null mouse fibroblasts were transfected with the expression vector containing the human wild type cDNA as positive control. Three stably transfected clones were selected: Pol β null/wt clone expressing human wild type Pol β , Pol β null/ Ex2 Δ clone and Pol β wt/ Ex2 Δ clone expressing Ex2 Δ Pol β variant.

RT-PCR confirmed the presence of the human Ex2 Δ mRNA both in wt and Pol β null mouse fibroblasts (data not shown). However, no translation products were detected by western blot analysis. As shown in Figure 3A, Pol β specific polyclonal antibody was able to detect purified full length and 8-kDa domain proteins (lane 1) as well as endogenous (lane 2 and lane 6) or transfected (lane 4) Pol β but did not reveal the presence of any truncated splicing products in either Pol β null/Ex2 Δ (lane 5) or Pol β wt/Ex2 Δ (lane 6) cell extracts. As expected, no signal was observed in Pol β null cell extracts (lane 3).

The lack of a detectable translation product could be explained by rapid degradation of the predicted small polypeptide. To test this hypothesis a vector expressing HA-tagged Ex2 Δ or wild type Pol β was transiently transfected into human AGS cells. After incubation in the presence or in the absence of the proteasome inhibitor MG132, cells were harvested for western blotting or fixed for immunofluorescence analysis. As shown in Figure 3B, by using anti-HA specific monoclonal antibody we were able to detect the HA-tagged wild type (lane 3) but not

Ex2Δ Pol β (lane 1) in AGS cell extracts. When cells were incubated in the presence of MG132, the lack of evidence of Ex2Δ Pol β translation (lane 2) allowed to exclude that this protein is rapidly degraded. As expected, under the same conditions, an accumulation of the transiently expressed HA-Pol β wild type was observed (lane 4). An increased level of endogenous Pol β was detected by the anti-Pol β antibody in both extracts from cells expressing either the wild type or the Ex2Δ Pol β when grown in the presence of MG132 (data not shown). The lack of Ex2Δ variant translation product was confirmed by immunofluorescence analysis. While the production of HA-tagged wild type Pol β (Figure 3C, upper panel) was clearly detectable in transfected AGS cells and the signal was enhanced in the presence of MG132 (Figure 3C, lower panel), lack of fluorescence was observed in the same cells transfected with HA-tagged Ex2Δ Pol β (Figure 3D, upper panel), even after incubation with MG132 (Figure 3D, lower panel).

Overexpression of Ex2Δ variant does not affect alkylating agent sensitivity and base excision repair capacity of stably transfected mouse cells

It has been hypothesised that the exon 2 skip splice variant evades the premature termination codon (PTC) by alternative translation initiation sites [30] downstream of the legitimate initiation site in exon 8, leading to the production of a truncated protein of 181 amino acids. Pol β truncated variants able to interfere with the wild type Pol β activity have been previously described [33]. To test whether the Ex2Δ variant is able to adversely affect the cell DNA damage response, we generated wild-type and Pol β null stable cell lines that carry an Ex2Δ variant transgene. Figure 4 shows that the expression of this transgene does not alter the sensitivity to the killing effect of the alkylating agent, methylmethane sulfonate (MMS), of the host cells. Panel A shows the response of Pol β null cells either transfected with a transgene expressing Ex2Δ variant (Pol β null/Ex2Δ cells) or the wild type Pol β (Pol β null/wt cells). Pol β null/wt cells were more resistant to MMS than Pol β null cells, indicating that the exogenous recombinant Pol β was able to complement, although partially, the sensitive phenotype of this cell line. In contrast, Pol β null/Ex2Δ were similarly hypersensitive to the cytotoxic effect of MMS as the untransfected Pol β null cells. Panel B shows the response of wildtype cells. Also in this case the expression of the Ex2Δ variant (wt/Ex2Δ cells) did not affect the survival of the host cells after MMS treatment.

Whole cell extracts of transfected clones were then tested in an *in vitro* Pol β gap filling assay [6]. Pol β null/wt cells were able to perform the 1 nucleotide (nt) DNA repair synthesis reaction (Figure 4C, lane 7–9), even if at lower efficiency than Pol β wt fibroblasts (lane 1–3). In contrast, Pol β null/Ex2Δ cells (lane 10–12) were unable to perform the 1nt gap-filling reaction as the parental Pol β null cells (lane 4–6). Pol β wt/Ex2Δ cells (lanes 13–15) showed similar gap-filling efficiency as the extract from wt un-transfected cells (lane 1–3).

All together, these observations indicate that the expression of Ex2Δ Pol β variant, that is the predominant Pol β splice variant in human cells, does not affect the cellular BER activity and response to alkylating agents.

Ex2Δ Pol β mRNA is more stable than the wild type transcript and localizes at polyribosomes

To check whether the most frequent Pol β splicing variant transcripts found in our samples were polyadenylated, RT-PCR on total RNA from AGS cells was performed by using oligo-dT. The pattern of bands obtained is consistent with polyadenylation of the predominant Pol β splicing variant transcripts (Figure 5A). The presence of polyadenylated Ex2Δ mRNA was confirmed by nested-PCR (data not shown). The stability of the Ex2Δ mRNA as compared with that of the wild type transcript was then addressed. The AGS cells were treated with actinomycin D to inhibit transcription, and then QPCR was performed to measure wild type and Ex2Δ mRNA decay. Surprisingly, while wild type Pol β mRNA half-life was confirmed

to be of the order of a few hours (<4 hrs), Ex2Δ Pol β mRNA was highly stable, with a half-life >8 hrs (Figure 5B).

The high frequency of Ex2Δ Pol β mRNA and its high stability led us to investigate if it was localised to sites of active translation on polyribosomes, by hypothesising a function as RNA cargo or post-transcriptional regulator independent from translation.

AGS cell lysates were fractionated and resolved on sucrose density gradients. Each gradient fraction was analysed by QPCR to detect wild type and Ex2Δ Pol β mRNA (Figure 5C). Both transcripts were present at high levels in the same fraction, indicating that they are complexed with the same number of ribosomes. As expected, the 18S mRNA, used as control of polysomes fractionation, was spread in many fractions.

The localization of Ex2Δ Pol β mRNA to polyribosomes suggests that this transcript may function to regulate translation of other genes. The potential regulation of Pol β itself was tested. A two-fold increase in Ex2Δ Pol β expression level did not induce any change in the levels of Pol β mRNA and protein (data not shown), leading us to conclude that other genes, different from Pol β, could be targets of this variant transcript.

4. DISCUSSION

GC has been described as a multistep process in which mucosal inflammation has a pivotal role [35]. The inflammatory process is characterised by a massive production of reactive oxygen species, that induces DNA damage that is preferentially repaired by BER. Overexpression of two DNA glycosylases that initiate BER has been described in association with ulcerative colitis, that is a risk factor for GC, and this adaptive increase of expression correlated with microsatellite instability [36]. Mutations in Pol β have been described in several tumor types such as colon, prostate, bladder and stomach (reviewed in [37]) and some of these mutations have been shown to lead to a mutator phenotype [24–25,38]. In the case of gastric cancer, a high frequency of mutations (approximately 30%) has been reported in patients from China [22] and Japan [23]. Two of these variants encode for functionally defective polymerases that either lack the DNA polymerase [25] or the dRpase [38] activity. In our set of gastric cancer specimens we found no evidence of Pol β mutations. This discrepancy might be related to population specific life style risk factors (e.g. dietary patterns, infection by *H. pylori*) that might be relevant for mutation type and frequency. Pol β splice variants were the most frequent event detected in our set of normal and tumor tissues and tumor cell lines. The simultaneous analysis of genomic and cDNA showed unambiguously that these are splice variants. Although splice variants and, in particular, the Ex2Δ variant, have been described more than a decade ago [22,31], the relative quantity and the functional role of alternative Pol β splicing variants have been poorly explored. Even in the case of the Ex11Δ variant that has been described to interfere with BER [33], its detection in both normal and tumor tissues leaves open the question as to its function.

Several lines of evidence have shown that Pol β splice variants are not a specific trait of cancer tissues. Here, we compare from a qualitative and, for Ex2Δ and Ex11Δ variants, from a quantitative point of view Pol β splicing variant pattern. We show that it is similar in tumor and matched normal mucosa from the same subject, confirming that it is not a unique feature of tumor tissues. A large inter-individual variability was observed in the relative frequency of different exon losses although the predominant event remained the deletion of exon 2. Ex2Δ splice variants also were detected with high frequency in gastric tissues and blood samples from healthy individuals. Exon 11 isoforms that include the Ex11Δ variant that act as dominant negative [33] were less frequent but they were detected in both normal and tumor tissues. These findings together with previous observations of Pol β splicing variants in normal tissues of

different origin [28–30,39–40] allow us to exclude the hypothesis that these skipping events are an hallmark of disease or that they are tissue-specific. The large inter-individual variability in the expression level of the splicing variants, as well as of Pol β reveals the complexity of the events that regulate Pol β gene expression.

The Ex2 Δ Pol β variant is the most represented splicing variant in our GC samples (74% of all variants detected), in agreement with results previously reported for bladder tissues [29], where Ex2 Δ cDNA represented 86% of sequenced cDNAs. Exon 2 was lost either alone or in combination with other exons, but exon 3 was retained in all cases. Taylor et al.[29] reported two variants generated by the loss of both exons in their set of bladder cancers but also in this study exon 3 was maintained in the majority of the cases.

Analysis by QPCR revealed that the Ex2 Δ mRNA isoform was produced in similar amounts as the wild type mRNA in normal and tumor tissues suggesting that common transcription factors and regulation might occur. By *in vitro* transcription/translation assay, Disher and Skandalis [41] reported that the Ex2 Δ mRNA is not translated. We provide the first *in vivo* evidence that Ex2 Δ variant mRNA does not encode protein, as detected by western blotting and immunofluorescence analysis (also in the presence of proteasome inhibitors) of human cells transiently transfected with HA-tagged Ex2 Δ variant and by DNA repair functional assays in Pol β null mouse cells expressing this variant. The experiments performed in the presence of proteasome inhibitors allowed us to exclude that the expected protein is rapidly degraded. Exon 2 loss also did not affect the cellular response to alkylating agents and the BER capacity of mouse cells overexpressing this variant. It has been hypothesized that the abundance of Pol β splicing variants might represent a post-transcriptional regulation mechanism [29]. This mechanism could be mediated by the production of Pol β variant truncated proteins that act as dominant negative factors (e.g. the variant lacking Ex11) [33] or inhibit Pol β by substrate competition [42]. We identify another possible regulatory mechanism, i.e. the production of ncRNAs. The production of non-functional isoforms of active genes is indeed a widespread mechanism for post-transcriptional gene regulation [43] known as Regulated Unproductive Splicing and Translation (RUST). These isoforms are usually degraded by non-sense mediated decay (NMD). In the case of Pol β some isoforms have been reported to follow this process but others, although containing a PTC (as in the case of Ex2 Δ), persist [41]. It has been demonstrated that the impairment of NMD can be induced by the presence of transcripts containing a PTC close to the AUG start codon [44]. This could apply to some Pol β isoforms.

Besides the evasion from NMD, this study revealed additional interesting features of Ex2 Δ transcript. It is polyadenylated, its half-life greatly exceeds that of wild type mRNA and it is localised at active sites of translation on polyribosomes like wild type transcripts. The half-life of a transcript can be affected by several factors such as translation rate, intracellular localization and even minor changes in sequence or mRNA structure (reviewed in [45]). It could be hypothesised that the wild type and the Ex2 Δ transcript have slightly different structure that withdraws different *trans*-acting regulator factors. All together the features of Ex2 Δ might be compatible with a role of this transcript as post-transcriptional regulator. Non coding transcripts can repress the transcription of coding genes by interacting with transcription factors, RNAP or promoter sequences [46]. We tested whether the regulation of Pol β in human cells was affected by Ex2 Δ mRNA overexpression. The lack of an effect both at transcription or at translation level, indicate that Pol β is an unlikely target for this ncRNA. It has been hypothesized that Ex2 Δ could regulate genes involved in oxidative stress response, since its frequency increases after oxidative damage [41]. Further studies are required to identify possible targets of this ncRNA.

In conclusion we provide definitive evidence that Pol β splicing variants are not markers of tumorigenesis in gastric tissues and are widely present in normal tissues likely being an intrinsic

feature of Pol β gene. Ex2 Δ transcripts are indeed conserved among many mammalian species (Ensembl Genome Browser). Their high frequency in several cells and tissues together with the lack of translation suggest that they could either regulate the levels of Pol β , that are critical for genome stability, by production of non functional isoforms or they could modulate target mRNAs involved in the cell response to endogenous/exogenous stresses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Ettore Meccia for helpful suggestions. Grant support: Associazione Italiana per la Ricerca sul Cancro (AIRC), MIUR/FIRB (RBNE01RNN7), Collaborative Project ISS/NIH. This research was supported in part by the Intramural Research Program of the National Institutes of Health, NIEHS.

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Fig. 1.

Pol β splicing variants in human gastric tissues and gastric cell lines (A) Analysis of Pol β cDNA by RT-PCR of mRNA from gastric tissues or cell lines. Primers were designed to amplify the entire Pol β coding sequence. Lane 1: DNA marker; lane 2: normal (N) and lane 3: tumor (T) tissues of one GC patient (code 88); lane 4: AGS gastric cancer cell line. (B) Nested-PCR of amplified Pol β cDNA from normal (N) and tumor (T) tissue of one GC patient (code 17). The 200 bp fragment represents the wild type amplicon. Lower molecular weight products indicate the occurrence of splicing events. The skipping of exon 2 (lane 1–2), exon 5 +6 (lane 3–4) and exon 9 (lane 5–6) is shown.

Fig. 2.

QPCR analysis of Pol β wild type and splicing variant mRNAs in normal and tumor tissues. Expression levels were measured in paired normal and tumor gastric specimens from 7 gastric cancer patients and gastric and blood samples from healthy individuals. (A) Pol β wild type, (B) Ex2 Δ and (C) Ex11 Δ isoforms mRNA levels in tumor tissues relative to paired normal tissues. (D) Ex2 Δ Pol β transcript levels in normal and tumor tissues relative to wild type Pol β mRNA levels in the same tissues. Gastric cancer patients are indicated by code. Specimens from healthy subjects are indicated as follows: gastric mucosa (N1 and N2) and blood samples (B1 to B4). The mean $\pm$ SD of three independent experiments is shown.

Fig. 3.

Western blot and immunofluorescence analysis of Pol β in mouse fibroblasts and human AGS cells. (A) Wild type and Pol β defective mouse fibroblasts were stably transfected with wt and Ex2Δ Pol β cDNA. Lane 1: Full length and 8-kDa domain Pol β recombinant proteins. Lane 2: Pol β wt cells. Lane 3: Pol β null cells. Lane 4: Pol β null cells transfected with human wild type Pol β. Lane 5: Pol β null cells transfected with the Ex2Δ Pol β variant. Lane 6: Pol β wt cells transfected with the Ex2Δ Pol β variant. (B) AGS cells were transiently transfected with HA-tagged wt and Ex2Δ Pol β cDNA. Lane 1: AGS cells transfected with HA-Ex2Δ Pol β. Lane 2: AGS cells transfected with HA-Ex2Δ Pol β and incubated with MG132. Lane 3: AGS cells transfected with HA-wt Pol β. Lane 4: AGS cells transfected with HA-wt Pol β and

incubated with MG132. Lane 6: AGS cells untransfected. All the lanes framed in the same box belong to the same autoradiography although some lanes between samples were cut because not relevant. (C) AGS cells transfected with HA-wt Pol β (upper panel) and incubated with MG132 (lower panel). (D) AGS cells transfected with HA-Ex2 Δ Pol β (upper panel) and incubated with MG132 (lower panel).

Fig. 4. Characterization of Pol β mouse cells transfected with the Ex2 Δ Pol β variant. Cell growth rate after MMS treatment of (A) Pol β defective cells: Pol β null ($\blacksquare$), Pol β null/wt ($\blacktriangle$), Pol β null/Ex2 Δ ($\circ$) and (B) Pol β wild-type cells : Pol β wt ($\bullet$) and Pol β wt/Ex2 Δ ($\square$) cells. Bars represent standard deviation. (C) Gap-filling assay performed with cell extracts from Pol β wt (lanes 1–3), Pol β null (lanes 4–6), Pol β null/wt (lanes 7–9), Pol β null/Ex2 Δ (lanes 10–12) and Pol β wt/Ex2 Δ cells (lanes 13–15). A typical experiment is shown.

Fig. 5. (A) Analysis of Pol β splicing variant polyadenylation. RT-PCR was performed on total RNA (1 μ g) from AGS gastric cancer cell line by using random examers (lane 1) or oligo dT (lane 2). Primers which recognize the entire coding sequence of Pol β cDNA were used in a second PCR to obtain Pol β splicing variants amplification. The position in the gel of the 1353 bp marker is shown. Multiple bands represent Pol β splicing variants. (B) Analysis of wild type and Ex2 Δ Pol β mRNA stability performed by QPCR on RNA of AGS gastric cancer cell line treated with actinomycin D. The mean $\pm$ SD of four independent experiments is shown. (C) Polyribosome distribution of wild type (—) and Ex2 Δ (---) Pol β mRNA. Human 18S (---) was used as positive control. The contents of each transcript were quantified by QPCR. The fraction with the highest level of transcript was used as reference (=1).

Table 3

Pol β splicing pattern of gastric tissues and cell lines.

Sample code *	Exon 2 **	Exon 5/6 §	Exon 5+6	Exon 9	Exon 12
N12	+	-	-	-	-
T12	+	-	-	-	-
N16	+	-	+	-	-
T16	+	-	+	-	-
N17	+	-	+	+	-
T17	+	-	+	+	-
N22	+	-	-	-	-
T22	+	-	-	-	-
N24	+	-	-	-	-
T24	+	-	-	-	-
N27	+	-	+	+	-
T27	+	-	+	+	-
N32	+	-	-	-	-
T32	+	-	-	-	-
N34	+	-	-	-	-
T34	+	-	-	-	-
N35	+	-	-	-	-
T35	+	-	+	+	-
N39	+	-	+	+	-
T39	+	-	+	-	-
N43	+	-	+	-	-
T43	+	-	+	-	-
N50	+	-	+	-	-
T50	+	-	-	+	-
N51	+	-	+	+	-
T51	+	-	+	-	-
N53	+	-	-	-	-
T53	+	-	-	-	-
N54	+	-	-	-	-
T54	+	-	-	-	-
N55	+	-	+	-	-
T55	+	-	+	-	-
N74	+	-	+	-	-
T74	+	-	+	-	-
N88	+	-	+	-	-
T88	+	-	+	-	-
N104	+	-	+	-	-
T104	+	-	+	-	-
N119	+	-	+	-	-
T119	+	-	+	+	-
AGS	+	+	+	+	-
NCI-N87	+	-	-	-	-
SNU-16	+	-	-	-	-

* N=normal, T= tumor tissue
** (+) indicates the occurrence of exon skipping
§ Since exon 5 and 6 present the same size, this analysis does not allow to assign the loss to a specific exon.

Table 4

Distribution of Pol β splicing variants in AGS gastric cancer cell line as determined by sequencing of Pol β cDNA clones.

Pol β splice variants	Splice variant frequency	PTC [*]	Putative protein length (amino acids)
Δ Exon 2	18/35	Yes	26
Δ Exon 2, Σ exon α	1/35	Yes	26
Δ Exon 2, 9	1/35	Yes	26
Δ Exon 2, 11	4/35	Yes	26
Δ Exon 2, 4–6, 11	1/35	Yes	26
Δ Exon 2, 4–11	1/35	Yes	26
Δ Exon 3–6, 9, 11	2/35	Yes	46
Δ Exon 11	1/35	No	306
Wild type	6/35	No	335

* PTC = Premature Termination Codon