

Role for nucleotide-excision repair gene variants in oxaliplatin-induced peripheral neuropathy

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ABSTRACT

PURPOSE:

Oxaliplatin forms part of routine treatment for advanced colorectal cancer (aCRC); however, it often causes severe peripheral neuropathy resulting in treatment discontinuation. We sought to determine the molecular and cellular mechanism underlying this toxicity.

PATIENTS AND METHODS:

We exome resequenced blood DNA samples from nine aCRC patients who had severe peripheral neuropathy associated with oxaliplatin (PNAO) within 12 weeks of treatment. We Sanger sequenced the *ERCC4* and *ERCC6* open reading frames in 63 patients with PNAO and carried out targeted genotyping in 1,763 patients without PNAO. We tested the functionality of *ERCC4* variants using viability and DNA repair assays in *Schizosaccharomyces pombe* and human cell lines after exposure to oxaliplatin and UV light.

RESULTS:

Exome resequencing identified one patient carrying a novel germline truncating mutation in the nucleotide excision repair (NER) gene *ERCC4*. This mutation was functionally-associated with sensitivity to oxaliplatin ($P=3.5 \times 10^{-2}$). We subsequently found that multiple rare *ERCC4* nonsynonymous variants were overrepresented in affected individuals ($P=7.7 \times 10^{-3}$) and three of these were defective in the repair of UV light-induced DNA damage ($P < 1 \times 10^{-3}$). We validated a role for NER genes in PNAO by finding that multiple rare *ERCC6* nonsynonymous variants were similarly overrepresented in affected individuals ($P=2.4 \times 10^{-8}$). Excluding private variants,

22.2% (14/63) of patients with PNAO carried Pro379Ser or Glu875Gly in *ERCC4*, or, Asp425Ala, Gly446Asp or Ser797Cys in *ERCC6*, as compared to 8.7% (152/1,750) of unaffected patients (OR=3.0, 95% CI 1.6-5.6, $P=2.5 \times 10^{-4}$).

CONCLUSIONS:

Our study provides evidence for a role of NER genes in oxaliplatin-induced peripheral neuropathy, together with mechanistic insights.

INTRODUCTION

Oxaliplatin, a third-generation platinum drug, in combination with 5-fluorouracil and leucovorin (FL), or oral capecitabine, is standard treatment for locally advanced and metastatic colorectal cancer (CRC); it improves both response and progression-free survival.^{1,2} It also improves disease-free survival in the adjuvant treatment of stage II and III colon cancer patients.³ In addition, oxaliplatin is widely used to treat other gastrointestinal malignancies. Platinum agents exert their effects by forming inter- and intra-strand DNA cross-links,⁴ which stall the cell cycle, inhibit DNA synthesis⁵ and trigger apoptosis.⁶ Oxaliplatin also induces oxidative DNA damage.⁷

Peripheral neuropathy is a well-recognised dose-limiting toxicity of oxaliplatin.^{8,9} High cumulative doses are associated with chronic peripheral nerve damage causing sensory ataxia and functional impairment.¹⁰ Chronic sensory neuropathy has been observed in around half of patients who received oxaliplatin with infusional FL.¹¹ Importantly, it is neurotoxicity, rather than tumour progression, which is often the cause of treatment discontinuation.¹² Since neurotoxicity is not correlated with response,¹² it is considered a potentially avoidable side effect. The underlying cause of peripheral neuropathy is not known, although oxidative stress may be a contributing factor.¹³⁻¹⁵

Although numerous genetic associations with peripheral neuropathy have been proposed (*GSTP1*,¹⁶⁻¹⁹ *AGXT*,²⁰ *ERCC1*,^{21,22} *FARS2*,²² *TAC1*,²² *SCN10A*,²³ *SCN4A*,²³ *VAC14*,²⁴ together with several genome-wide associated loci^{25,26}), none have been independently validated and introduced into patient stratification.

Here, we sought to delineate the underlying cause by exome resequencing patients with severe peripheral neuropathy after treatment with oxaliplatin-based chemotherapy.

MATERIALS AND METHODS

Patients

We analysed blood DNA samples from unrelated patients with advanced CRC (aCRC) from the UK national trial COIN (NCT00182715).²⁷ Patients were randomised to receive continuous oxaliplatin and fluoropyrimidine chemotherapy, continuous chemotherapy plus cetuximab, or intermittent chemotherapy. In all patients, treatment was identical for the first 12 weeks apart from the choice of fluoropyrimidine together with the randomisation of \pm cetuximab. All patients gave fully informed consent for their samples to be used for bowel cancer research (approved by REC [04/MRE06/60]). We obtained the maximum grade of peripheral neuropathy after 12 weeks of treatment. Patients with grade 3/4 peripheral neuropathy or that had had oxaliplatin-dose reduction due to severe peripheral neuropathy were classified as suffering from peripheral neuropathy associated with oxaliplatin (PNAO). Patients with no, or grade 1, peripheral neuropathy formed a control group and were classified as not having PNAO. Patients with grade 2 peripheral neuropathy were excluded to allow a better discrimination between the two patient groups.

Molecular analyses

We excluded known inherited neuropathies by carrying out multiplex ligation-dependent probe amplification analysis of *PMP22* (~75% of patients with Charcot-

Marie-Tooth syndrome, the most common form of inherited neuropathy, have a 1.4MB duplication) and by examining the exome resequencing data for *PMP22* and 65 other genes associated with rare inherited neuropathies (*Supplemental Materials and Methods*).

Library fragments containing exomic DNA were collected using the Roche Nimblegen SeqCap EZ Exome Library solution-based method. Massively parallel sequencing was performed on the Illumina Genome Analyser. On average, across the exome, we had 55% (range 46-60%) coverage of the open reading frame (ORF) at 20-fold depth. Fastq files were processed through a sequence analysis pipeline using BWA for sequence alignment and modules from the Broad Institute's Genome analysis Toolkit to recalibrate quality scores, refine alignments around potential insertions/deletions (indels), eliminate duplicate reads, call indel and SNP genotypes, generate QC metrics and apply quality filters to the genotype calls. SNP calls were annotated using ANNOVAR. PCR and Sanger sequencing were carried out as described in *Supplemental Materials and Methods*. *ERCC4* and *ERCC6* nonsynonymous variants were genotyped using KASPar (LGC) or BeadArray (Illumina) technologies (*Supplemental Materials and Methods*).

Functional analyses

Production of a *Schizosaccharomyces pombe rad16* base strain, the *rad16* wild type vector, *Cre* recombinase mediated cassette exchange, transformation of the base strain, site-directed mutagenesis and treatment with oxaliplatin and UV light was carried out as described in *Supplemental Materials and Methods*. 480 EBV-transformed human lymphoblastoid cell lines established from healthy Caucasian

individuals (ECACC, Salisbury) were assayed for the *ERCC4* variants Pro379Ser, Arg576Thr and Glu875Gly using KASPar (LGC). Three cell lines for each variant in a heterozygous state and wild type controls (n=3) were selected for the functional analyses. Cell lines were established in RPMI 1640 supplemented with 10% foetal bovine serum, penicillin/streptomycin and L-glutamine and maintained at 37°C and 5.0% CO₂. Survival analyses were carried out as described in *Supplemental Materials and Methods*. For DNA repair assays, cells were irradiated with 70J UV-C and aliquots removed at 0, 4, 24 and 48 hours after treatment, sorted by FACS for viable cells, and DNA extracted. DNA samples were probed for CPDs using an ELISA kit (Cell BioLabs) and absorbance was read at 450nm using a plate reader, with a reference range of 620nm (*Supplemental Materials and Methods*).

Statistical and bioinformatic analyses

For association analyses, R v.3.3.2 was used for the Pearson's Chi-squared test or Fishers exact test, where appropriate. Average survival data for oxaliplatin and UV light exposure in *S.pombe* was normalised to wild type and analysed using SPSS v.23 ANOVA with Dunnett correction (following transformation using the arcsine function). For DNA repair assays, statistical analyses were performed in SPSS using a two-way ANOVA, with mutation status and treatment as the independent variables. The dependent variable was CPD quantification (ng/ml) as a measure of DNA repair. Individual ANOVAs were run at 24 and 48 hours. *In silico* predictions for functional significance of nonsynonymous variants were determined using Align-Grantham Variation/Grantham Deviation (Align-GVGD). Linkage disequilibrium (LD) was obtained using Haploview v.4.2.

RESULTS

Of the 2,445 patients with aCRC in the COIN trial, 23% of those that received oxaliplatin and fluorouracil-based therapy and 16% of those that received oxaliplatin and capecitabine-based therapy had severe (\geq grade 3) peripheral neuropathy over the course of the trial.²⁷ We focussed on patients with severe PNAO within the first 12 weeks of treatment (*Supplemental Materials and Methods* and Table 1) as a potentially enriched group for causal germline mutations (n=63 patients). Although fewer of these patients responded to treatment at 12 weeks (47%, 26/55 with response data; Table 1) as compared to patients without PNAO (grade \leq 1, n=1,763; 57%, 884/1542 with response data), this was not statistically significant ($P=0.14$).

Nine of the 63 patients with severe PNAO had exome resequencing of their germline blood DNA samples. These patients were selected based on review of their medical notes and had no potential confounding clinical complications. We identified on average 48 (range 40-56) stop gains and 88 (73-111) indels predicted to result in frameshift mutations, per patient exome (*Supplemental Table*). We excluded known inherited neuropathies in these patients by *PMP22* dosage analysis and by examining the resequencing data for 66 candidate genes (no stop gains or truncating indels were predicted).

Novel truncating mutation in *ERCC4*

Variants not present in dbSNP v.132 (assigned as novel) were considered most likely to cause PNAO; we identified on average 8 (range 2-11) and 28 (16-57) novel stop gains and frameshifting indels, respectively, per patient (*Supplemental Table*). We also considered that germline truncating mutations in genes involved in

oxaliplatin transport, metabolism or the repair of its associated damage might be responsible for PNAO; we identified 104 such genes from literature reviews (*Supplemental Materials and Methods*). All nine patients carried truncating variants in these selected genes (range 1-4); however, only one of these variants, in a single patient, was novel (*Supplemental Table*). Patient 8 carried the novel stop gain Ser613X in the nucleotide excision repair (NER) gene *ERCC4*, which was confirmed by Sanger sequencing of an independent PCR product (*Supplemental Table*). We did not find any other coding region variants in the second *ERCC4* allele in Patient 8 after direct sequence analysis of their entire ORF and flanking intronic sequences. Clinical review confirmed that this patient did not have xeroderma pigmentosum (XP) (caused by bi-allelic *ERCC4* mutations).

We carried out a more comprehensive analysis of all known DNA repair genes (REPAIRtoire, n=163 genes, <http://repairtoire.genesilico.pl/>,²⁸ and, MD Anderson / Wood's DNA repair list, n=244 genes, <https://www.mdanderson.org/documents/Labs/Wood-Laboratory/human-dna-repair-genes.html>,²⁹), including those in the base excision repair system that repair oxidative DNA damage,³⁰ but did not find any further novel stop-gains or truncating indels.

Functional analysis of the *ERCC4* stop mutation

We investigated whether the *ERCC4* nonsense mutation induced sensitivity to oxaliplatin and UV light (causes cyclobutane pyrimidine dimers [CPDs] that are repaired by NER). We re-created the mutation in the *Schizosaccharomyces pombe* homolog *rad16* (Ser585X) in a base strain and a strain deficient in endonuclease

uve1 (a *pombe*-specific alternative UV light repair system). Following oxaliplatin treatment, we observed decreased survival for *rad16-Ser585X* ($P=3.5 \times 10^{-2}$) in comparison to wild type *rad16* (*rad16*⁺), and in a similar range to a control *rad16* deleted mutant (*rad16* Δ) (Fig.1A). Similarly, we observed decreased survival of *uve1* Δ -*rad16-Ser585X* following treatment with UV light ($P < 1 \times 10^{-3}$) (Fig.1B).

Multiple rare *ERCC4* variants associated with peripheral neuropathy

We sought further evidence for a role of *ERCC4* in PNAO and Sanger sequenced the *ERCC4* ORF and flanking intronic sequences in all 63 patients with PNAO. We did not find any further stop gains or truncating indels; however, we did identify four rare (minor allele frequencies <5% in dbSNP) nonsynonymous variants (Pro379Ser, rs1799802, in 3 patients; His466Gln, rs372950439, in 1 patient; Arg576Thr, rs1800068, in 1 patient; Glu875Gly, rs1800124, in 4 patients) (Table 1). Pro379Ser, Arg576Thr and Glu875Gly were predicted to interfere with function (Table 2). We also identified one common nonsynonymous (Arg415Gln, rs1800067), three synonymous and three 5' untranslated region (UTR) variants. We genotyped the *ERCC4* nonsynonymous variants in all COIN patients with available samples. His466Gln was not seen in any further patients. Each of the other rare variants were found more frequently in cases with, as compared to those without, PNAO (Table 2). Combined, significantly more patients with PNAO carried one of these variants (13.1% [8/61] as compared to 5.2% [87/1,671] of patients without PNAO; $P=7.7 \times 10^{-3}$).

The common variant Arg415Gln was found in similar proportions of patients with (14.3% [9/63]) and without (14.8% [260/1,754]) PNAO ($P=0.87$). An intronic variant in *ERCC4* (rs1799800), associated with late onset bortezomib-associated neuropathy³¹,

was not associated with PNAO (found in 38.1% of patients with PNAO as compared to 48.0% without, $P=0.12$).

Functional analysis of *ERCC4* nonsynonymous variants

We sought evidence for causal effects of Pro379Ser, Arg576Thr and Glu875Gly using EBV-transformed human lymphoblastoid cell lines established from healthy individuals that carried each variant in a heterozygous state ($n=3$ for each variant and wild type controls). Although treatment with UV light reduced viability in all lines, we did not observe any differences between wild type and variant cell lines (data not shown). In terms of repair capacity after DNA damage with UV light, all wild type cell lines showed noticeable repair 24 hours after treatment, with the majority of CPDs being repaired by 48 hours (Fig.2). In contrast, all three sets of variant cell lines displayed reduced repair in the initial ($P<1\times 10^{-3}$ at both 24 and 48hours) and validation ($P<1\times 10^{-3}$ at both 24 and 48hours) experiments (Fig.2).

Validated role for NER in PNAO

We attempted to validate a role for NER gene defects in PNAO and sought novel nonsynonymous variants in all *ERCC* gene family members by re-analysing the exome resequencing data. We identified Gly929Arg in *ERCC6* in one patient, which was confirmed using an independent PCR product. We sought further potentially causal *ERCC6* variants by direct sequence analysis of the ORF, intronic boundaries and 5'UTR in all 63 patients with PNAO. We identified nine rare (Table 1) and five common nonsynonymous variants, two synonymous variants and one 5'UTR variant; we genotyped nonsynonymous variants in all available cases. Seven rare nonsynonymous variants were predicted to be damaging (C55-C65), three of which

(Asp425Ala, Pro694Leu and Ser797Cys) were individually overrepresented in patients with PNAO (Table 3). Combined, rare *ERCC6* nonsynonymous variants were highly associated with peripheral neuropathy (in 20.6% [13/63] of patients with PNAO as compared to 4.7% [82/1749] of patients without, $P=2.4 \times 10^{-8}$) (Table 3).

No common *ERCC6* nonsynonymous variants were associated with PNAO: Gly399Asp was in (patients with, versus those without, PNAO) 27.0% versus 30.7%, $P=0.54$; Arg1213Gly in 36.5% versus 34.6%, $P=0.76$; Arg1230Pro in 22.2% versus 19.3%, $P=0.57$; and, Gln1413Arg in 36.5% versus 34.5%, $P=0.74$.

Combined analyses of *ERCC4* and *ERCC6*

Since private variants may skew statistical associations, we considered only rare *ERCC4* and *ERCC6* nonsynonymous variants that were present in ≥ 2 patients with PNAO. In total, 22.2% of patients with PNAO carried Pro379Ser or Glu875Gly in *ERCC4*, or, Asp425Ala, Gly446Asp or Ser797Cys in *ERCC6*, as compared to 8.7% of unaffected patients (OR=3.0, 95% CI 1.6-5.6, $P=2.5 \times 10^{-4}$) (Table 4).

DISCUSSION

The rare variant hypothesis predicts that individually rare, but collectively common, inherited variants play a significant role in disease susceptibility.³² For example, rare nonsynonymous variants in the genes encoding apolipoprotein A1, the adenosine triphosphate binding cassette transporter A1 and lecithin cholesterol acyltransferase, are over-represented in individuals with low plasma levels of high-density lipoprotein cholesterol, a major risk factor for coronary atherosclerosis.³³ Furthermore, multiple rare nonsynonymous variants in *APC* play a significant role in inherited

predisposition to colorectal adenomas.³⁴ Here, after identifying a novel *ERCC4* truncating mutation in a patient with PNAO, we found that multiple rare *ERCC4* and *ERCC6* nonsynonymous variants were over-represented in affected individuals. Therefore, the rare variant hypothesis may also be applicable to germline susceptibility to toxicity from therapy. If validated by others, the *ERCC4* and *ERCC6* nonsynonymous variants described *herein* would be considered ‘moderately’ penetrant risk alleles for oxaliplatin-induced peripheral neuropathy, as a proportion of carriers did not have PNAO within 12 weeks of treatment.

ERCC4 forms a complex with *ERCC1*, which carries out 5' incision of damaged DNA in NER, the main repair pathway involved in the removal of bulky and DNA-distorting adducts.³⁵ The complex has also been implicated in interstrand crosslink (ICL) repair³⁶ and in the repair of double strand breaks.³⁷ *ERCC6* encodes CSB, a SWI/SNF DNA-dependent related ATPase;³⁸ it is recruited to areas of DNA damage following stalling of RNA polymerase II and has multiple roles including chromatin remodelling³⁹ and recruitment of other NER proteins.⁴⁰ Given that *ERCC4* and *ERCC6* are likely targets of peripheral neuropathy-associated nonsynonymous variants, thorough examination of other NER genes is warranted to determine whether they play similar roles in toxicity to oxaliplatin.

Our finding that NER genes may play a role in oxaliplatin-induced peripheral neuropathy, is supported by observations from several other studies. Firstly, patients with XPA, XPC, XPG and Cockayne syndrome related disorders (caused by biallelic *ERCC* mutations) suffer from peripheral neuropathy prior to treatment.⁴¹ The majority (17/20) of our *ERCC4* or *ERCC6* carriers had only one locus-specific mutant allele,

and, to our knowledge, none had XP or Cockayne syndrome group B, suggesting that haploinsufficiency for a mutant allele may be sufficient to induce peripheral neuropathy upon exposure to oxaliplatin. Secondly, an *Ercc1*^{-Δ} murine model, which has reduced expression of the ERCC4-ERCC1 complex, develops accelerated spontaneous peripheral neurodegeneration with significant structural alterations of the sciatic nerves.⁴² Third, in *Xpa*^{-/-} and *Xpc*^{-/-} mice, chronic exposure to cisplatin resulted in an accelerated accumulation of unrepaired ICLs in neuronal cells.⁴³ Furthermore, the augmented adduct levels in dorsal root ganglion cells of these mice coincided with an earlier onset of peripheral neuropathy-like functional disturbance of their sensory nervous system.

Few predictive biomarkers for toxicity to therapy in the treatment of CRC have been independently validated. Two rare variants in *DPYD* have been associated with severe toxicity in patients receiving 5-FU⁴⁴ and a polymorphism in *UGT-1A* has been linked to a higher risk of developing irinotecan-associated neutropenia and diarrhea;⁴⁵ however, none of these biomarkers have been introduced into routine clinical practice due to their poor sensitivity and specificity. Here, we identified roles for NER gene variants in toxicity to oxaliplatin, which, if validated, may represent an opportunity for patient stratification.

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AUTHOR CONTRIBUTIONS

JPCheadle obtained funding for this study. The study was designed by JPCheadle with input from MJW, HLM, TSM, RAA, MC, HW, and OF, and was carried out under the direction of JPCheadle. JPColley undertook the exome resequencing under the guidance and facilities of MJW and HLM. MJW undertook the exome bioinformatic analyses. HW undertook the literature searches, Sanger sequencing, validation analyses and genotyping with support from RH. HW carried out the functional analyses in *S.pombe* under the guidance and facilities of OF. MC carried out the functional analyses in human cells. HW and MC undertook the statistical analyses. TSM was CI of COIN, and, RAA was a COIN trial fellow; both provided clinical advice and assistance. RSK managed the COIN trial and, with DF, facilitated access to the clinical data. JPCheadle and HW interpreted the data with input from co-authors. JPCheadle and HW wrote the paper with input from MC, and all authors provided comments.

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Table 1. Clinical information and, *ERCC4* and *ERCC6* genotypes, for 63 patients with severe PNAO

ID	Age	Sex	Arm	Chemotherapy	Response at 12 weeks	Max grade PN (12 weeks)	Oxaliplatin stopped for neurotoxicity within 12 weeks?	If stopped, time from randomisation (days)	<i>ERCC4</i>					<i>ERCC6</i>									
									Pro379Ser	His466Gln	Arg576Thr	Ser613X	Glu875Gly	Asp425Ala	Gly466Asp	Pro694Leu	Ser797Cys	Gly929Arg	Phe1217Cys	Ala1296Thr	Phe1437Ile	Thr1441Ile	
1	49	F	C	OxMdG	Partial response	3	Y	33	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
2	73	M	B	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n
3	68	F	C	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
4	72	F	A	OxMdG	Stable disease	3	Y	41	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
5	61	M	C	XELOX	Stable disease	3	Y	27	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
6	77	M	B	XELOX	No assessment	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
7	73	M	C	OxMdG	Progressive disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n
8	77	F	A	XELOX	No assessment	3			n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n
9	76	F	C	XELOX	No assessment	3			n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n
10	52	F	A	OxMdG	Stable disease	3	Y	59	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
11	62	M	C	OxMdG	Partial response	3	Y	49	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
12	59	F	C	OxMdG	Stable disease	3	Y	62	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
13	62	M	B	XELOX	Progressive disease	3	Y	79	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
14	71	F	B	XELOX	Partial response	4			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
15	56	F	B	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
16	48	M	B	XELOX	Progressive disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
17	77	M	A	XELOX	Stable disease	3	Y	79	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
18	69	M	C	XELOX	Stable disease	3	Y	67	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
19	62	M	B	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
20	69	F	A	XELOX	Partial response	3	Y	64	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
21	65	F	A	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
22	73	F	B	XELOX	Complete response	3	Y	63	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
23	45	F	A	XELOX	Stable disease	3	Y	84	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
24	57	M	A	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
25	68	M	B	OxMdG	Partial response	3	Y	55	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
26	70	M	C	XELOX	No assessment	3	Y	77	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
27	69	F	C	XELOX	No assessment	3	Y	59	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
28	63	M	B	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
29	76	F	B	XELOX	Partial response	3	Y	73	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
30	73	M	B	XELOX	Partial response	3	Y	66	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
31	73	F	C	XELOX	Partial response	3	Y	64	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
32	75	M	A	OxMdG	Partial response	3			n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
33	77	F	A	OxMdG	Stable disease	3	Y	76	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n

34	73	F	B	OxMdG	Stable disease	3	Y	54	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
35	40	F	A	OxMdG	Progressive disease	3	Y	52	n/n	n/n	m/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
36	55	F	A	OxMdG	Stable disease	3	Y	79	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
37	69	F	A	XELOX	Partial response	3			n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
38	78	M	B	XELOX	No assessment	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
39	67	M	C	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
40	65	M	B	OxMdG	No assessment	3	Y	78	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
41	69	M	B	XELOX	Stable disease	4			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
42	64	M	B	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
43	77	F	A	XELOX	Stable disease	3	Y	76	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
44	60	F	B	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	m/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
45	73	M	B	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n
46	68	M	A	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
47	53	F	A	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
48	73	M	B	XELOX	Complete response	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
49	56	F	A	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
50	40	F	C	XELOX	Partial response	3	Y	63	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
51	65	F	A	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	m/m
52	67	F	B	XELOX	Stable disease	3	Y	48	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
53	73	M	B	XELOX	Stable disease	3	Y	70	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
54	74	F	C	XELOX	Partial response	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
55	63	F	B	XELOX	Partial response	3	Y	49	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
56	78	M	B	XELOX	Partial response	3	Y	67	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
57	62	F	A	OxMdG	Partial response	3	Y	68	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
58	61	M	A	XELOX	Stable disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
59	50	F	A	XELOX	Progressive disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
60	74	F	C	XELOX	Progressive disease	3			n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
61	77	M	C	OxMdG	Partial response	3	Y	55	n/n	n/n	n/n	n/n	n/n	n/n	n/n	m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
62	40	M	B	XELOX	No assessment	3			m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n
63	48	M	A	OxMdG	Partial response	3			m/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n	n/n

Patients 1-9 were exome resequenced and those highlighted carried variants. COIN Arm A - received continuous oxaliplatin and fluoropyrimidine chemotherapy, Arm B - continuous chemotherapy plus cetuximab, Arm C - intermittent chemotherapy. Patients had infusional 5FU or capecitabine as the combination partner with oxaliplatin (OxMdG and XELOX, respectively). Abbreviations - PN, peripheral neuropathy; m, mutant; n, normal allele.

Table 2 – Rare *ERCC4* nonsynonymous and stop-gain variants in patients with (+) and without (-) PNAO.

Variant	rs number	^a Align-GVGD score	Frequency in patients		χ^2	P
			+ PNAO	- PNAO		
Pro379Ser	rs1799802	C65	3/63 (4.8%)	27/1,763 (1.5%)	-	0.08
His466Gln	rs372950439	C15	1/61 (1.6%)	0/1,677 (0%)	-	-
Arg576Thr	rs1800068	C65	1/63 (1.6%)	4/1,762 (0.2%)	-	0.16
Ser613X	Novel	C65	1/63 (1.6%)	-	-	-
Glu875Gly	rs1800124	C65	4/63 (6.4%)	60/1,763 (3.4%)	-	0.28
^b Total			8/61 (13.1%)	87/1,671 (5.2%)	7.1	7.7x10⁻³
excluding private variants			7/63 (11.1%)	86/1,763 (4.9%)	4.9	2.7x10⁻²

^aScore of predicted functional impact by Align-Grantham Variation/Grantham Deviation (Align-GVGD): C65 – most likely and C15 – less likely to affect function.

^bSer613X was not included in the total since it was only assayed in cases with PNAO; one patient with PNAO carried Arg576Thr and Glu875Gly, and another without carried Pro379Ser and Glu875Gly. Values reflect the number of patients successfully genotyped (totals - for all variants).

Table 3 - Rare *ERCC6* nonsynonymous variants in patients with (+) and without (-) PNAO.

Variant	rs number	^a Align-GVGD score	Frequency in patients		<i>X</i> ²	<i>P</i>
			+ PNAO	- PNAO		
Asp425Ala	rs4253046	C65	3/63 (4.8%)	15/1,763 (0.9%)	-	0.02
Gly446Asp	rs4253047	C65	3/63 (4.8%)	55/1,750 (3.1%)	-	0.45
Pro694Leu	rs114852424	C65	1/63 (1.6%)	0/1,763 (0%)	-	0.03
Ser797Cys	rs146043988	C65	2/63 (3.2%)	2/1,763 (0.1%)	-	0.01
Gly929Arg	Novel	N/A	1/63 (1.6%)	1/1763 (0.1%)	-	0.07
Phe1217Cys	rs61760166	C65	1/63 (1.6%)	3/1,763 (0.2%)	-	0.13
Ala1296Thr	rs139509516	C55	1/63 (1.6%)	1/1,762 (0.1%)	-	0.07
Phe1437Ile	rs758679804	C15	1/63 (1.6%)	3/1,763 (0.2%)	-	0.13
Thr1441Ile	rs4253230	C65	1/63 (1.6%)	4/1,763 (0.2%)	-	0.16
^b Total			13/63 (20.6%)	82/1,749 (4.7%)	31.1	2.4x10⁻⁸
excluding private variants			7/63 (11.1%)	71/1,750 (4.1%)	5.7	1.7x10⁻²

^aScore of predicted functional impact: C65 – most likely, C55 – likely, C15 – less likely to affect function; N/A – not applicable (alternative transcript). ^bOne patient with PNAO carried Asp425Ala and Ser797Cys, and, one patient without PNAO carried Asp425Ala and Gly446Asp, and another carried Gly446Asp and Phe1217Cys. Values reflect the number of patients successfully genotyped (totals - for all variants).

Table 4 – Combined analysis of non-private, rare *ERCC4* and *ERCC6* nonsynonymous variants.

Gene	Variants	Frequency in patients (%)		X ²	OR (95% CIs)	P
		+ PNAO	- PNAO			
<i>ERCC4</i>	Pro379Ser, Glu875Gly	7/63 (11.1%)	86/1,763 (4.9%)	4.9	-	2.7x10⁻²
<i>ERCC6</i>	Asp425Ala, Gly446Asp, Ser797Cys	7/63 (11.1%)	71/1,750 (4.1%)	5.7	-	1.7x10⁻²
	^a Total	14/63 (22.2%)	152/1,750 (8.7%)	13.4	3.0 (1.6-5.6)	2.5x10⁻⁴

^aFour patients without PNAO carried Glu875Gly in *ERCC4* and Gly446Asp in *ERCC6* and another carried Pro379Ser in *ERCC4* and Gly446Asp in *ERCC6*.

LEGENDS TO FIGURES

Figure 1. The *ERCC4* nonsense mutation induced sensitivity to oxaliplatin and UV light in a *S. pombe* (*rad16*) model system. Average percentage survival from (A) four independent experiments following oxaliplatin treatment, or, (B) three independent experiments following treatment with UV light, for a control *rad16* gene deletion mutant (*rad16Δ*) and *rad16-Ser585X* (mimics Ser613X seen in a patient with PNAO), normalised to *rad16⁺*. For treatment with UV light, all strains were *uve1Δ*. Standard deviations displayed as vertical bars.

Figure 2. *ERCC4* nonsynonymous variants displayed reduced repair capacity. Average CPD concentrations (ng/ml) after UV-C (70J) irradiation in wild type cells and cells carrying (A) Pro379Ser, (B) Arg576Thr and (C) Glu875Gly over a 48hr period, showing the initial (*top panels*) and validation (*lower panels*) experiments. CPD concentrations are plotted as an average of two duplicate samples from the same experiment run on separate ELISA plates, which are shown against time with standard error bars (data from the wild type cells is shown in A, B and C). The test and validation experiments were biological repeats. V = variant cell line; C = wild type control cell line; 1-3 = different cell lines.