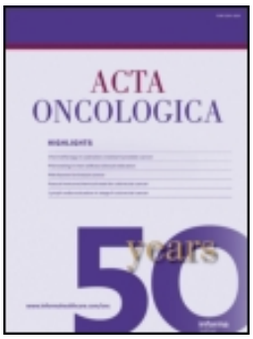

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ORIGINAL ARTICLE

p53 mutation and cyclin D1 amplification correlate with cisplatin sensitivity in xenografted human squamous cell carcinomas from head and neck

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Abstract

To investigate the response of tumour growth to cisplatin treatment, in relation to p53 mutation and cyclin D1 dysregulation on DNA and protein level, biopsies from seven xenografted human squamous cell carcinomas from the head and neck were analysed with immunohistochemistry for p53 expression and cyclin D1 expression. Polymerase chain reaction - singlestranded conformation polymorphism was used to determine p53 mutations. Fluorescence in situ hybridization was performed to analyse cyclin D1 amplification. The mice were injected i.p. with NaCl (controls) or cisplatin. After injection the tumour volume were measured. The inhibition of tumour growth by cisplatin was defined as the area under the growth curves, and compared with the growth curves of the tumours in the control group. Xenografts with p53 mutation showed significantly higher resistance to cisplatin ($p < 0.001$) and also tumours with cyclin D1 amplification showed significantly higher resistance ($p < 0.001$).

Cisplatin is an alkylating chemotherapeutic drug used to treat several types of cancer, including squamous cell carcinoma of the head and neck (SCCHN). However, the response to cisplatin in SCCHN varies widely from total response to almost complete resistance. Cognetti et al. [1] showed improved survival of patients with SCCHN who responded completely to cisplatin treatment. In a randomized study, Beauvillain et al. [2] similarly demonstrated chemoresponse to be the most important prognosticator in patients with advanced laryngopharyngeal cancer. Better outcome with concomitant chemo and radiotherapy has recently been demonstrated [3] while, neoadjuvant or adjuvant chemotherapy, combined with conventional therapy did not show improved survival [4]. The effectiveness of treatment with cisplatin is restricted by intrinsic or acquired cellular resistance. The exact mechanisms governing cisplatin cellular uptake and inactivation have not been identified [5]. The cellular response to cisplatin-DNA adducts, with respect to DNA repair, apoptosis, and/or cell cycle arrest, has been investigated yielding many contradictory results. One of the central genes in this context is p53. This gene is a transcription factor and activates downstream regulator genes in response to DNA damage i.e. p21 [6]. We and others have shown a correlation between poor prognosis for patients with SCCHN and p53 mutation [7,8]. A correlation has also been found between p53 mutation and cisplatin-fluorouracil neoadjuvant chemotherapy resistance [9]. In contrast, Bradford et al. [10] found that p53 mutation was correlated to cisplatin sensitivity in vitro. The cyclin D1 gene, CCND1, another cell cycle regulator, is frequently overexpressed and amplified in SCCHN. This is associated with poor prognosis [11]. The aims of this study were to investigate the response of tumour growth to cisplatin treatment, in relation to p53 mutations and cyclin D1 dysregulation on DNA and protein level.

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Methods and material

Tumours

To establish xenografted squamous carcinomas cell lines (SCC) biopsies were taken under general anaesthesia from patients with SCC not previously treated. The biopsies were then divided into two pieces, one for xenografting and one for in vitro cultivation. This study was approved by ethics committee.

The biopsies were xenografted to nude mice and serially passed as previously described [16]. The cell lines studied originated from the following subsites: one from the larynx, one from the base of the tongue, four from the oral cavity and one from a lymph metastasis with the primary tumour unknown (Table I).

To test the effect of chemotherapy, cell grafting was performed when the mice were 5 – 8 weeks old with a xenograft on each flank. Tumours were allowed to grow approximately two weeks by which time they had reached a sufficient volume and were in the growth phase.

Tumour volume and weight measurements

Three days before, and on the day of treatment the animals were weighed and the tumour volume measured, to ensure that no weight loss had occurred and that an appropriate tumour volume had been reached. Tumour volumes less than 40 mm^3 and greater than 300 mm^3 were excluded, as well as animals showing weight loss. The tumour volume was calculated from orthogonal diameter measurements every two or three days using the formula: volume = length × $width^2$ /2 [17]. The mice were weighed regularly during treatment

Treatment

On the day of cisplatin treatment, mice carrying the same tumour line were divided into four groups: the control group A was injected i.p. with NaCl; group B was injected i.p. with 2.5 mg/kg cisplatin body weight; group C 5.0 mg/kg and group D 7.5 mg/kg cisplatin body weight. The administered doses were diluted with saline to the required concentration and injected i.p. in volumes of 0.01 – 0.015 ml b.wt.

Table I. Tumour origin and tumour line flow cytometry

Tumour line LU-HNxSCCX	Site of primary tumour origin	Ploidity	% s phase	DNA Indices
14	Oral cavity	Non-diploid	6.2	1.81
7	Bucca	Non-diploid	18.2	1.56
11	Primary tumour unknown	Non-diploid	16.6	1.33
12	Larynx	Non-diploid	4.8	1.31
5	Gingiva	Non-diploid	13.2	2.21
8	Bucca	Non-diploid	2.0	1.80
4	Oral cavity	Non-diploid	10.6	1.63

Cisplatin induced tumour growth inhibition

The tumour volume at day n relative to the tumour volume at the start of treatment (RTS) was transformed in to a ^{10}log value in order to obtain a normal distribution. The area under the logRTS growth curve vs. time (AUC) was determined according to Lesser [18]. The logRTS growth curves for the control group showed exponential growth and the doubling time of the tumour was determined. The AUC was calculated from the day of treatment to the day when the control group tumours had reached tripled in volume. A relative AUC value was calculated according to the formula: Relative AUC = AUC for the actual tumour /mean AUC value for the controls.

Immunohistochemistry

Untreated xenografts were allowed to grow for approximately two weeks, to reach sufficient size and to be in growth phase. Specimens were embedded in paraffin and immunohistochemical (IHC) analysis was performed to detect cyclin D1 and p53 expression. Commercial monoclonal antibodies were used (Novo, NCL-cyclin D1) at a dilution of 1:20 and p53 clone D0-7, Dako A-C Denmark, at a dilution of 1:300. A strongly positive specimen was used as a positive control. The IHC results (Table II) were scored as follows: A-negative; B 1 – 5% of the tumour cells positive; C 6 – 50% positive; D >50% positive. The negative controls were tested without primer antibodies.

Fluorescence in situ hybridization

FISH, fluorescence in situ hybridization was performed as earlier described [19] with minor modifications. Briefly, touch imprints from xenografted tumour cells were made on Superfrost Plus slides and then air-dried. These imprints were fixed in Carnoy's solution (3:1 methanol:acetic acid) for 10 minutes and air-dried for one hour. The imprints were denatured in a solution of (70% formamide, 2xSSC, pH 7) at 72°C for three minutes and then dehydrated in a series of ethanol solutions (70%, 85% and 100%).

Two-colour hybridization was performed with a labelled gene-specific probe for CCND1 and with a labelled chromosome 11 centromere probe. A hybridization mixture containing the probe (LSI Cyclin D1 spectrum orange TM/CEP 11 spectrum

green TM DNA Probe, 33-191039,vysis), 0.8 μl unmarked placenta DNA (D3287, Sigma) and 6.8 μl hybridization buffer (30-804826, vysis) was denatured in 72°C for 5 minutes and then hybridized at 37°C in a chamber overnight. After hybridization, the excess of the probe was washed away using (0.4xSSC, 0.3% Nonidet p40 at 72°C for 2 min; 2xSCC for 1min and then distilled water). DAPI (4,6-diamino-2-phenylindole) was added to a solution (Vectashield, Vector H1000) to visualize the DNA and to prevent the fluorochromates from fading. Zeiss fluorescence microscope was used to analyse the samples. The amplification rate was calculated as gene probe/centromere probe = copy number and was required to be over 1.5 to be considered as amplified. Tumour cell line no. 5 showed pronounced amplification and therefore marked with +++.

PCR-SSCP and DNA sequencing

Polymerase chain reaction-single strand conformation polymorphism analysis (PCR-SSCP) and DNA sequencing, were used to study the occurrence of p53 mutations in exons 4 – 11, as previously described [20]. Briefly, DNA was extracted using standard methods and used in a polymerase chain reaction to amplify the entire coding region of the p53 gene in seven or eight different fragments. The PCR products were screened for mutations by SSCP. Samples showing altered mobility shift in SSCP were further analysed by direct DNA sequencing to determine the exact location and type of mutation.

Flow cytometry (FCM)

FCM analysis was performed according to Wennerberg et al. [16]. Briefly, the tumours were minced, forced through a nylon net (pore size 140 μm, Tidbeck AB, Stockholm, Sweden), and fixed in 70% ethanol. The separated cells were thereafter exposed to ribonuclease (Sigma-Aldrich, Stockholm, Sweden), incubated with pepsin solution (Merck, Darmstadt, Germany), and then stained with propidium iodide (Sigma-Aldrich, Stockholm). Human lymphocytes were processed in parallel with the tumour samples and used as an external diploid controls. Flow cytometric DNA analysis was performed in a FACS Caliber (Bacton, Dickinsen, Biosciences). DNA content was measured using an argon laser and up to 20 000 nuclei were analysed in each sample. The DNA histograms obtained were automatically processed using Modfit LT 3.1 software. The DNA indices (DI) were calculated as ratios of the modal channel values of G0/G1- peaks. By definition, the tumours manifesting a single DNA population were classified as diploid (with DI = 1.00), and tumours manifesting two or more populations as non-diploid. The s-phase fraction (SPF) was estimated assuming that s-phase compartment constituted a rectangular distribution between the modal values of G0/G1 and G2 peaks.

Statistics

Data were analysed with the RS/1 data analysis system. The normality of the distribution was tested with the Wilk-Skapiro test. Differences between groups were tested with one-way analysis (ANOVA) and the Kruskal-Wallis test.

Results

Expression of the p53 gene

As can be seen in Table II, five out of seven tumour cell lines had p53 mutations; one of these had two different mutations. The mutations were located in exons 4, 5, 6 and 7. Three mutations were missense type (amino acid substitution), one nonsense, one

Table II. Description of p53 mutation, cyclin D1 dysregulation and relative AUC in xenografts. The immunohistochemical results were scored: A negative; B 1–5% of the tumour cells positive; C 6–50% positive; D >50% positive.

	p53 mut.	Amino acid	Type of mutation	IHC p53	FISH cycline D1	IHC cycline D1	Rel. AUC mean
LU-HNxSCX 14	–	–	–	A	–	B	0.19
LU-HNxSCX 7	–	–	–	A	–	B	0.29
LU-HNxSCX 11	Exon 6	Arg 196 ter Arg 72 Pro*	Nonsense	A	–	B	0.40
LU-HNxSCX 12	Exon 4	Ter 122	Frame shift	A	+	B	0.69
LU-HNxSCX 5	Exon 5+7	His 168 Pro 11e 254 Asn	Missence	D	+++	D	0.94
LU-HNxSCX 8	Exon 7	Leu 252 del	Inframe deletion	D	+1/3**	C	0.77
LU-HNxSCX 4	Exon 7	Arg 249 Gly	Missence	D	+	B	0.70

*contain also the R72P polymorphism.

**two cellpopulations were observed. One tumour cell population was FISH positive and represented 1/3 of all tumour cells in sample.

a frame- shifting single base pair deletion and one a frame-three base pairs deletion. A previously described, a polymorphic variant (Arg72Pro) was also detected. Two xenografts had p53 mutations in introns +72c >t and they were regarded as negative samples in our study. The three cell lines with missense or inframe-deletion mutations also showed p53 overexpression according to IHC, while the two cell lines with truncating mutations had no detectable protein expression. As expected, the two cell lines without p53 mutation had no p53 overexpression.

Cyclin D1 expression

Four xenografted tumours showed CCND1 gene amplification, two of which also revealed overexpression of cyclin D1, as determined by IHC (Table II). Three of the tumours showed no CCND1 amplification and no protein overexpression. Two tumour lines showed CCND1 amplification but no overexpression of the protein. All tumour lines with CCND1 amplification also had p53 mutation.

Cisplatin sensitivity

To compare the cisplatin sensitivity of the different tumour lines the dose of cisplatin 2.5 cisplatin mg/kg body weight was chosen. This low dose was chosen to better discriminate the differences in cisplatin sensitivity, since high doses effect both sensitive and resistant xenografts. The area under the growth curve was calculated and related to that of the controls (relative AUC). A higher relative AUC value indicates a higher resistance to cisplatin. A value of 1.0 indicates no growth inhibition as a result of treatment with cisplatin.

Figure 1 shows the sensitivity of the different tumour lines. The tumour lines LU-HNxSCC 4, 5, 8, 11, 12 with p53 mutations showed significantly lower cisplatin sensitivity than tumours with wt p53 ($p < 0.001$). Similarly, the tumour lines with CCND1 amplification showed significantly lower cisplatin sensitivity than those without amplification ($p < 0.001$). Cisplatin sensitivity was then compared between tumour lines with both p53 mutations and CCND1 amplification.The tumour line with only p53 mutations, showed significantly greater cisplatin sensitivity than that with both p53 mutations and CCND1 amplification ($p = 0.004$).

Flow cytometry

As can been seen in Table I, all the tumour lines were non-diploid, with s-phase fraction of the tumour cells between 2.0 and 18.2%. There was no correlation between s-phase fraction and cisplatin sensitivity.

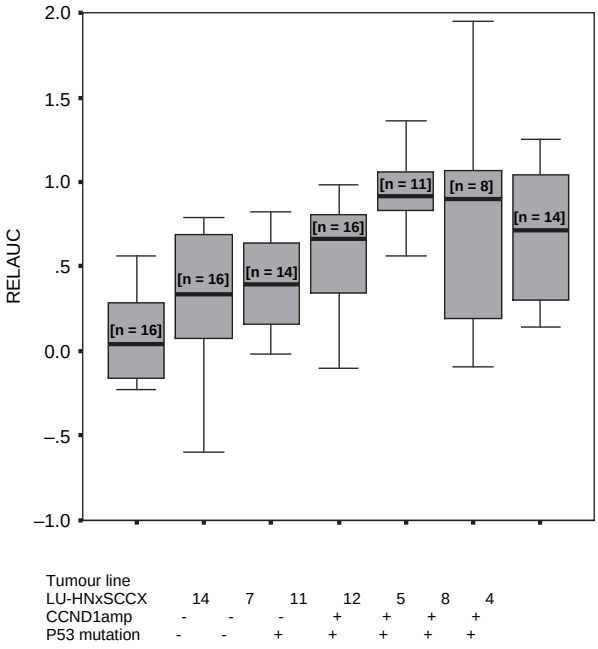

Figure 1. Boxplot diagram of relative AUC in various tumour lines, showing the 2½, 25, 50, 75 and 97½ cumulative relative frequencies. (n =number of tumour lines).

Discussion

Our results show that squamous cell carcinoma tumour lines from the head and neck without cyclin D1 gene amplification or p53 mutation respond better to cisplatin treatment (Figure 1). DNA-sensing genes such as ATM, ATR or DNA-PK activate p53 by phosphorylation, by which p53 can induce cell cycle arrest or apoptosis. Missense or contact mutations of p53 have been related to poor prognosis [8] and radioresistance in SCCHN in both clinical studies [23] and in vitro models [24]. Similarly, an increased sensitivity to cisplatin in patients with wtp53 has been reported [9], although the converse has been demonstrated [10]. P53 mutations have also been associated with increased radiosensitivity [25]. The conflicting results could be due to differences in cell background, propensity for apoptosis and co-expression of oncogenes. Recently, additional proteins, such as p63 and p73, have been found to interact with p53 and to be involved in apoptosis and cell cycle regulation. A p53 polymorphism at codon 72, encoding either arginine (72R) or proline (72P), influenced cisplatin-based chemo-radiotherapy in advanced SCCHN, in that cancers expressing 72R mutants showed a lower response rate than 72P mutants. A possible explanation of this could be the involvement of p73-dependent apoptosis [26]. A polymorphism has been noted in one tumour cell line, proline at codon 72, a tumour line that also had a nonsense mutation.

We can not draw any conclusions regarding cisplatin response, from this finding.

The cyclin D1 gene/protein has been implicated in tumourogenes because of its importance in regulating G1 to S-phase checkpoint and consequently promoting cell cycle progression. Overexpression of cyclin D1 has been shown to stimulate cell proliferation [27] and tumour progression [28]. Thus, the use of antisense cyclin D1 in vitro in human squamous carcinoma, including head and neck, results in decreased cell proliferation, suppressed growth and the induction of apoptosis [29]. Akervall and collaborators [12] have reported that cyclin D1 overexpression indicated better response to neoadjuvant treatment with cisplatin and 5-fluoro-uracil. They have also shown that cyclin D1 overexpression in cell lines is associated with better response to cisplatin, however, no correlation was observed between cyclin D1 gene amplification and cisplatin sensitivity [14]. In contrast, Ishiguro et al. [13] have found an inverse relationship between cyclin D1 gene amplification and response to neoadjuvant treatment of SCCHN with cisplatin and 5-FU. A decrease in the expression of cyclin D1 results in a better response to cisplatin treatment in a variety of human cancers [29] including SCCHN. Furthermore, studies have shown that antisense cyclin D1 enhances cisplatin sensitivity [15]. The role of cyclin D1 gene amplification in cisplatin sensitivity could not be determined from our data in this in vivo setting, as none of the tumour lines showed only cyclin D1 gene amplification without p53 mutation.

Further investigations and more standardized methods are needed to find the key to DNA repair, cell arrest and apoptosis. Despite the small number of animals, our results imply there is a correlation between cisplatin response and the dysregulation of CCND1 and p53. Further studies are required with several xenografts and a more extended investigation of a combination of multiple genes.

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