A Molecularly Genetic Determination of Prognostic Factors of the Prostate Cancer and Their Relationships to Expression of Protein p27^{kip1}

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Loss of a part of chromosome 8p22, containing the gene LPL and amplifications of the field 8q24 comprising the c- myc oncogene are the most frequent chromosomal aberrations in prostate cancer. We aimed to find the frequency of these chromosomal abnormalities and assess their relationship to the prognosis of prostate cancer patients in relation to Gleason score and expression of p27$^{Kip1.}$ We chose a subgroup of 17 monitored patients who had died during five years following diagnosis, and a group of 31 surviving patients whose Gleason score exceeded 5 (Group of Gleason score 2–3). Owing to lack of tumor cells in puncture biopsies, we made hybridizations in situ and objectively evaluated the result in 35 patients out of 48. Amplification in the field for oncogene c-myc was found in 19 cases (54.2%), in 15 of these (78.9%) polyploidy of the chromosome 8 was also confirmed. Deletion of a part of chromosome 8p22 was found in 21 cases (60%). Normal findings were shown in 8 cases (22.8%) and genetic abnormalities were revealed in 27 patients (77.2%). A Chi-squared test showed the dependence of Gleason score on amplification of the c-myc gene in nonmetastasing prostate cancer. In the case of a Gleason score of group 3, abnormalities in amplification of c-myc were statistically more significant than a Gleason score of group 2 using significance according to the Fisher Exact Probability test (p=0.039). We showed that the level of expression of protein p27 ^{Kip1} was related to abnormality of amplification of the c-myc gene. We also came to the conclusion that decreased expression of protein p27 ^{Kip1} is related the c- myc amplification.

Key words: 8p22 loss, 8q24 amplification, prostate cancer, Gleason 's score, p27^{Kip1}, prognosis

Prostate cancer is a heterogeneous disease with varying biological behavior. Frequent immunohistochemical examination aimed at clarifying the idiosyncratic behavior of this cancer is currently supplemented by the contributions of molecular genetics. The aim of our work was to utilize the conclusions of the immunohistochemical examination of ^{p27kip1} expression (hereinafter just p27), and compare them with the molecular genetic abnormalities most frequently occurring in carcinoma of the prostate. Progression of this tumor is known to be connected with a number of genetic abnormalities. Most frequently affected is chromosome 8 while deletions of the field 8p22 and amplifications of zone 8q24 are also frequent chromosomal alterations[1,2,3]. Changes in locus 8p22 where the gene for the tumor suppressor LPL (lipoprotein lipase) is situated are associated with poor prognosis [3] in patients with advanced prostate cancer (Fig.1). Loss of heterozygosity described in up to 69% of prostate cancers is responsible for the frequent changes in behaviour of prostate cancer. Part of the 8q24 chromosome is a zone in which the c-myc, oncogene which regulates cell proliferation and apoptosis, is localized. Amplification of this gene is more frequent in cases of tumors with a higher Gleason score [1,4]. Published studies report that amplification of the c-myc gene is invariably connected to immunohistochemical overexpression of the myc protein. It is hypothesised that excessive expression of the myc protein causes decreased expression of protein p27 which results in activation of the path of cyclin E/cyclin-dependent kinase 2 and consequent cell proliferation. The above knowledge base led us to evaluate the relation of genetic abnormalities to expression of protein p27, as explained in our previous work [5].

Materials and Method

The studied group consisted of 48 patients divided into: (a) a sub group of 17 patients from a set of monitored patients who had died in the 5 years following a primary diagnosis of nonmetastasising prostate cancer. Their Gleason score group

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(GSG) was 1–3 and samples were retrospectively immunohistochemically examined [5] and, (b) a subgroup of 31 surviving patients with a Gleason score group 2 or 3. From the total set of tumors, only 35 cases were successfully hybridized. All biopsy findings of prostate cancer were classified according to WHO [6], and histological grading was made, including determination of Gleason score grouping as 1,2 or 3 (See Table 1).

Table 1: Groups of patients according to Gleason's score (GS groups)

GS Group	Gleason's Score	Carcinoma Grading
1	By 5	Well dif. carcinoma
2	By 7	Intermediate dif. carcinoma
3	By 0 l0	Poorly dif. carcinoma

Table 2: A Summary of Findings at Individual Cases of Expression of p27, Group of the Gleason's Score and FISH.

p27 %positive coders	Group of Gleason's score	Deletion 8p22	Amplification 8q24	Polynomy 8 chromosome
5	2	No	Yes (AI)	No (monosomy)
5	2	Yes	No	No
0	2	Yes	No	No
0	2	Yes	No	No
25	3	Yes	Yes	Yes
25	3	Yes	Yes	Yes
5	2	Yes	No	No
0	3	Yes	Yes	Yes
25	2	Yes	Yes	Yes
5	2	No	Yes	Yes
75	3	No	No	No
75	2	No	No	No
5	3	Yes	Yes	Yes
50	3	Yes	Yes	Yes
5	2	No	Yes	Yes
0	2	No	Yes (AI)	No (monosomy)
50	2	No	No	No
25	2	Yes	No	No
50	2	No	No	No
25	3	Yes	Yes	Yes
0	2	Yes	Yes	Yes
75	2	No	No	No
50	2	Yes	No	No
50	2	Yes	No	No
50	2	Yes	No	No
75	2	No	No	No
0	1	Yes	Yes	No
0	1	No	No	No
25	3	Yes	Yes	Yes
5	1	No	Yes	Yes
50	1	No	No	No
50	2	Yes	Yes	No
50	1	Yes	Yes	Yes
0	2	No	Yes	Yes
0	2	Yes	Yes	Yes

To determine amplification of zone 8q24 or deletion of zone 8p22 fluorescence in situ hybridization (FISH) was performed on paraffin sections using probes (Pro Vision Multi-Color Probe,Vysis, Abbott, USA), which comprised three differently marked regions of the DNA chromosome 8. Thin cuts were made and treated as follows: incubation in 0.2 M HCl at room temperature for 20 minutes, incubation in a solution of 1M NaSCN (sodium thiocyanate), 98–102% titration at 80°C(Sigma, USA), soaking with protease (protease II, Vysis, Abbott, USA) at 37°C for 95 minutes, fixation in 10% buffered formalin at room temperature for 10 minutes, and drying on a heating plate at 45–50 °C. Probes and DNA of the sample were simultaneously denatured at a temperature of 85°C for 1 minute and then incubated for 8–24 hours at a temperature of 37°C in a wet cabinet. After hybridization, the excess and unhybridized probe were washed in 0.4x SSC (salt sodium citrate, Vysis, Abbott, USA) / 3% NP (nonylphenylpolyethylene glycol, Vysis, Abbott, USA) and buffered at a temperature of 73°C for 2 minutes. Samples were then washed in a 2xSSC/0.1% NP solution at room temperature for 1 minute and given the instability of fluoro-chromium, dried in a dark place. After core coloring using DAPI II (4.6diamidino-phenylindole, Vysis, Abbott, USA), the samples were covered with a cover slip. At least 100 separate cores of tumor cells were evaluated. A number of signals was defined for each core for zone 8p22, centromeres of chromosome 8 and zone 8q24.

The evaluation. A normal finding : < 10% cells with 3 or more signals and < 55% cells with 1 or 0 signals for CEP 8. A loss of CEP 8: > 55% cells with 1 or 0 signals for CEP 8. A loss of 8p22: a ratio of signals for 8p22/CEP8 <0.85. A loss of c-myc: ratio of signals for c-myc/CEP8 < 0.9. AI (additional increase, i.e. multiplication of number of gene copies which was not accompanied by adequate multiplication of number of chromosomes): a ratio of signals for c-myc/CEP8 >1.3 and >10% cells with 3 and more signals for c-myc. The expression of protein p27 was evaluated as ≤ 25 % cells as significantly reduced , 25% ≤ 50% as rather reduced, and > 50% as normal (Fig.2,3).

Statistical evaluation. Statistical analysis of the findings was carried out using a Chi-squared test, Pearson's correction and ANOVA using the SPSS Program, version 8.0**,** utilizing the significance of Fisher's Exact Probability Test. Tests for Tables higher than 2*2 were performed by a Chi-squared test (or by a Fischer's test with simulation of a p value), contingent tables 2*2 by the exact Fisher's test. The R-plus[7] Program version 2.3.0. was used for testing.

Results

The findings of hybridization and expression of protein p27 (Fig.4,5) are presented in Table 2.

From a total number of 48 hybridized cases, 35 cases were successfully hybridized, from which we observed amplifica-

tion of zone 8q24 in 19 cases (54.2 %), and in 15 of these (78.9%) also polysomy of chromosome 8. Deletion in zone 8p22 was found at 21 cases (60%). A normal finding was revealed in 8 cases (22.9%) and pathological findings were reported in 27 patients (77.1%). The relation of Gleason score group to individual genetic abnormalities is shown in Tables 3 and 4. A summary of the occurrence of both deletion 8p22 and amplification of 8q24 in relation to Gleason score group is presented in Table 5.

The maximum occurrence of genetic changes as deletion 8p22 was recorded in the GSG 3 (87.5%). The least occurrence was recorded in the GSG 1 (40%). However, the Chi-squared test with a p=0.163 showed there was no statistically significant relation of Gleason score to deletion 8p22.

The occurrence of genetic abnormality was 8q24 amplification, i.e. c-myc gene, was recorded in 87.5% cases in GSG

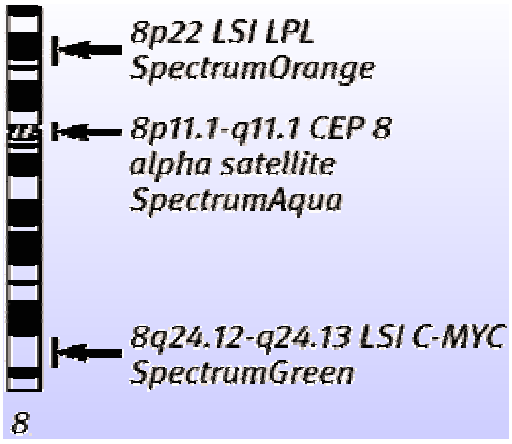

Figure 1. Scheme of chromosome 8

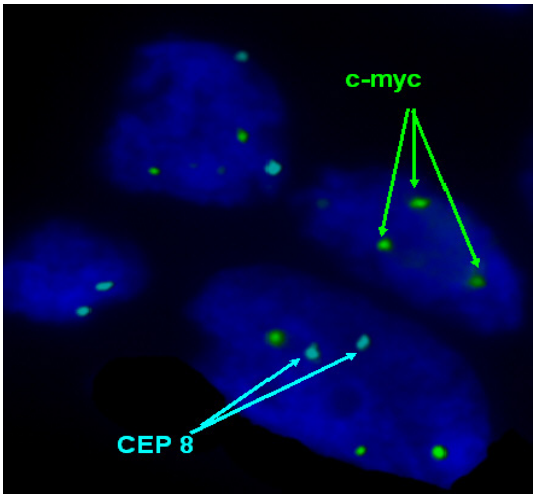

Figure 2. Normal number of c-myc gene (8q24), Olympus BX61, 1250x CCD -1300QB camera

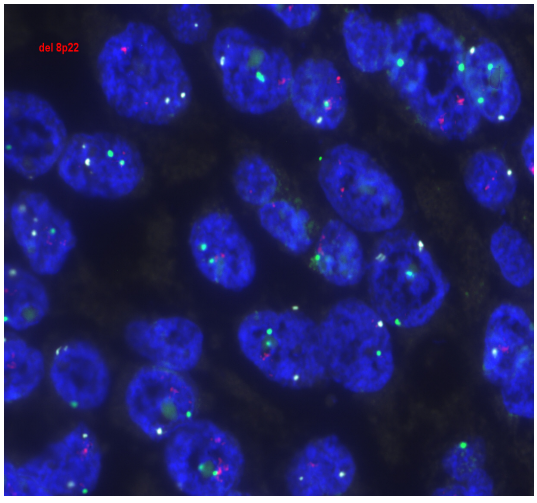

Figure 3. Deletion of gene LPL (8p22) Olympus BX61, 1250x, CCD -1300QB camera

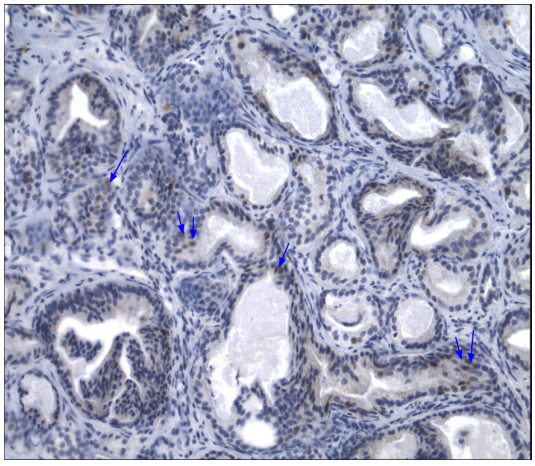

Figure 4 Expression of protein 27<25%, Olympus AX 70Provis, HE 10x20 Olympus 4040

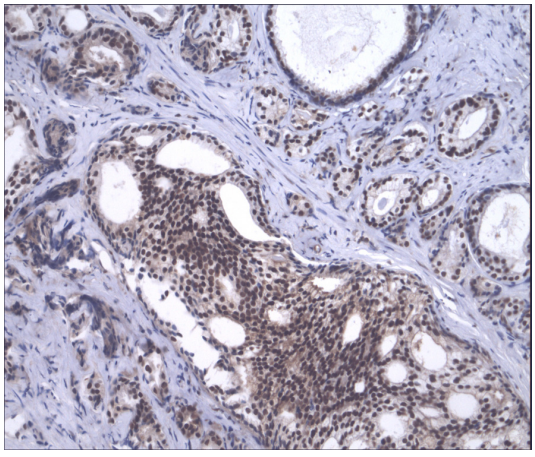

Figure 5. Expression of protein p27>75%, Olympus AX 70Provis, HE 10x20 Olympus 4040

o3. Surprisingly, the number of cases without amplification of c-myc gene was higher in group 2 (59.1%).

The finding of c-myc amplification in GSG 1 was higher than that with no amplification. However, the differences are less than for GSG 3.

The overall significance from the Chi-squared test was $p < 0.001$ showing the relation of Gleason score to amplification of c-myc gene. In the case of the Gleason score group 3, the occurrence of abnormalities of amplification of c-myc was statistically much more significant than that for a Gleason score group 2, where significance of the exact Fisher's test was p=0.039.

These findings indicate a direct relation of amplification of the gene c-myc to degree of differentiation of prostate cancer. From 19 amplifications, 16 cases (84%) are included in the GSG 2 or 3.

Genetic abnormalities were found in 87.5 % of cases in the GSG 3. In accordance with current suppositions, this reflects the reality of a larger number of genetic abnormities in poorly differentiated prostate adenocarcinomas. A similar situation also occurs at intermediate degrees of differentiation where genetic changes are recorded in 77.3%. The difference in genetic changes in % drops in the group with a GSG 1. However, the Chi-squared test with a p=0.517 shows the lack of significance of these relations. In this point, the very small numbers within the samples sizes should be noted. Another problem may be that the analysis was carried out on a relatively small number of tumor cells since it was not always possible to meet the requirement of 300 examined tumor cells due to their lack in tissue roller from puncture biopsy.

The findings of relation of protein p27 expression and genetic abnormalities of the type, deletion of 8p22 and amplification of 8q24 are presented in Tables 6 and 7. The relation of expression of the protein p27 and occurrence of monitored genetic abnormalities (deletion of 8p22 and amplification of 8q24) is presented in Table.8.

The findings of relation of protein p27 expression and amplification of c-myc gene are summarised in Table 7.

A decreased expression in protein p27 represented as an expression below 25% was reported in 16 cases (72.7%) which corresponds to 84.2% of all 19 cases with amplification of 8q24, i.e. the c-myc gene. Simultaneously, we also recorded 6 cases with no amplification which corresponds to 37.5% of all 16 carcinomas without genetic changes and abnormalities, and 27.3 % of all carcinomas with decreased expression of protein p27 (p=0.009). Protein p27 expression is statistically significantly related to the occurrence of amplification of c-myc. The relation between individual groups is : ≤ 25% versus 25% ≤ 50% – p=0.056, ≤ 25% versus > 50% – p=0.014, 25% ≤ 50% versus > 50% – p=0.578. In the case of p27 ≤ 25%, amplification of the c-myc gene is significantly higher than in the case of > 50%. The difference between the expres-

Table 3: A Gleason's score and occurrence of abnormality of deletion 8p22

Group of Gleason's score	Deletion occurrence 8p22	No deletion occurrence 8p22	Total
1	**2**	**3**	**5**
	40,0%	60,0%	100,0%
2	**12**	**10**	**22**
	54,5%	45,5%	100,0%
3	**7**	**1**	**8**
	87,5%	12,5%	100,0%
Total	**21**	**14**	**35**
	60,0%	40,0%	100,0%

Table 4: A Gleason's score and occurrence of genetic abnormality of the 8q24 amplification

Group of Gleason's score	Amplification occurrence 8p24	No amplification occurrence 8p24	Total
1	**3**	**2**	**5**
	60,0%	40,0%	100,0%
2	**9**	**13**	**22**
	40,9%	59,1%	100,0%
3	**7**	**1**	**8**
	87,5%	12,5%	100,0%
Total	**19**	**16**	**35**
	54,3%	45,7%	100,0%

Table 5: Gleason' score and genetic abnormalities as both deletion of 8p22 and amplification of 8q24.

Group of Gleason's score	With occurrence of genetic abnormalities	Without occurrence of genetic abnormalities	Total
1	**3**	**2**	**5**
	60,0%	40,0%	100,0%
2	**17**	**5**	**22**
	77,3%	22,7%	100,0%
3	7	1	8
	87,5%	12,5%	100,0%
Total	**27**	**8**	**35**
	77,2%	22,8%	100,0%

Table 6: The relation of p27 expression and amplification of 8q24

Expression p27	With occurrence of amplification of 8q24	Without occurrence of amplification of 8q24	Total
≤ 25%	**16**	**6**	**22**
	72,7%	27,3%	100,0%
≤ 50%	**3**	**6**	**9**
	42,8%	85,7%	100,0%
> 50%	**0**	**4**	**4**
	0%	100,0%	100,0%
Total	**19**	**16**	**35**
	54,3%	45,7%	100,0%

sion p27 ≤ 25% and 25% ≤ 50% is the limit of statistic significance.

A finding of decreased expression of p27 was recorded in carcinomas with deletion of 8p22 as well as without it. Deletion of 8p22 was not found in the group with expression of p27 higher than 50%. The significance of the Chi-squared test was p=0. 033. This shows the dependence of expression of and occurrence of genetic abnormality of deletion of 8p22.

The decreased expression of p27 represented as low expression (0–25%) was reported in 21 cases, which corresponds to 95.5% of cases of genetic abnormalities and 96.2% of all monitored genetic changes. A low and little reduced expression of p27 was recorded also in prostate carcinoma with occurrence of genetic abnormalities in 6 cases, which corresponds to 66.7%. Any case of prostate carcinoma was reported expression of protein exceeding 50%. A Chi-squared test with a p=0.0019 shows the dependence of expression of p27 and genetic abnormalities.

Discussion and conclusions

Recent studies have indicated that several chromosomes such as 7,8,10 and Y play important roles in tumor genesis and tumor progression in cases of prostate cancer [8,9,10,11]. Chromosome 8 alteration, including loss of 8p21–22 and gain of 8q24, are commonly observed. In our report, 35 (72.9%), out of a total number of 48 samples were successfully hybridized.- From these we observed amplification of zone 8q24 in 19 cases and in 15 of these polysomy of chromosome 8 as well. This, however, was reported both in a group of poorly differentiated adenocarcinomas, where the Gleason 's score exceeded 7 (GSG 3), as well as in cases of a well-differentiated adenocarcinoma with a Gleason score below 5 (GSG 1). Deletion of zone 8p22 was found in 21 cases, while in 7 cases the Gleason score was reported higher than 7 (GSG 3). A normal finding was proved in 8 cases. Genetic abnormalities were reported at 27 patients (77.1%). Our findings are in agreement with the literature according to which findings of amplification of the c-myc gene and LPL deletion are related to level of tumor differentiation and this has significant prognostic value (1,2,3,4). Pathological amplification and deletion according to the literature reach 66.6% of all cases. Oncogene amplification is one mechanism leading to stepwise progression in solid tumors. Published studies dealing with prostate carcinoma report that amplification of the gene c-myc is closely related to excessive expression of protein Myc. There is an assumption that excessive expression of the Myc protein causes of decreased expression of protein p27 which results in activation of pathway of cycline E/cycline-dependent kinase 2 and consequent cell proliferation. Therefore it is assumed that the c-myc gene deregulates cell growth and causes the increased proliferation of prostate carcinoma. Gene c-myc is evaluated as a marker of malignant prostate carcinoma potential.

Recently it has been shown that the level of expression of protein p27 relates to the Gleason score, tumor recurrence and survival time of patients with prostate carcinoma [1,8,9]. The relationship to genetic abnormalities has also been demonstrated in our study. A major problem is also interpretation of expression of p27 and its decreased expression which can be demonstrated by relocation of expression from nucleus into cytoplasm of tumor cells [12]. Our findings are in accordance with the literature [1,2,3,4]. Decreased expression of protein p27 represented by its low values prevails in a group of carcinomas where chromosomal changes are observed. A finding of significantly decreased expression of protein p27 is observed in carcinomas with amplification of the c-myc gene in 84.2% of all 19 carcinomas with amplification of c-myc gene. The Chi-squared test showed the clear dependence of Gleason score and amplification of the c-myc gene. In the GSG 3, the occurrence of abnormalities of amplification of c-myc gene was statistically more significant than for Gleason score group 2 (significance of the exact Fisher's test p=0.039). The relation of decreased expression of protein p27 to chromosome abnormalities was statistically significant, too (p=0.0019), deletion 8p22 (p=0.033) as well as to amplification of gene c-myc (p=0.009). A relatively large group of prostate carcinomas with decreased expression of p27 without amplification of gen c-myc (27.5%) suggests of other potential ways of degradation of expression of protein p27. Hower, to confirm and prove this would require a larger sample.

Table 7: The relation of p27expression and deletion of 8p22

Expression p27	With occurrence of deletion 8p22	Without occurrence of deletion 8p22	Total
≤ 25%	**15**	**7**	**22**
	68,2%	31,8%	100,0%
≤ 50%	**6**	**3**	**9**
	66,7%	33,3%	100,0%
> 50%	**0**	**4**	**4**
	0%	100,0%	100,0%
Total	**21**	**14**	**35**
	60,0%	40,0%	100,0%

Table 8: The relation of p27 expression and the followed genetic abnormalities in the prostate carcinoma (deletion of 8p22 and amplification of 8q24)

Expression p27	With occurrence of genetic abnormalities	Without occurrence of genetic abnormalities	Total
≤ 25%	**21**	**1**	**22**
	95,5%	4,5%	100,0%
≤ 50%	**6**	**3**	**9**
	66,7%	33,3%	100,0
> 50%	**0**	**4**	**4**
	0%	100,0%	100,0%
Total	**27**	**8**	**35**
	77,1%	22,9%	100,0%

This report suggests that amplification and overexpression of c-myc alone with another gene(s) mapped to 8q, may play a key role in the progression and prognosis of prostate cancer. On the other hand, the work confirms the suggestion of Matsuyam who notes the important role of deletion of chromosome 8p22 in the progression of prostate carcinoma . Cases with more than pT3 tumors had a significantly higher frequency of 8p22 deletion than those with pT2 ($p<0,01$). Studies on chromosomal deletions of 8p22 by the FISH technique may serve as a universal genetic marker to optimize the treatment strategy together with above mentioned abnormities in patients with prostate cancer [11]. The problems of observing the mutual relations of expression of proteins responsible for occurrence and development of prostate carcinoma and incidence of genetic abnormities in this disease will be further discussed. It is apposite to note some problems occuring during processing. One problem was that analysis was carried out with a relatively small number of tumor cells since it was not always possible to reach a requirement of 300 examined tumor cores due to their lack in the tissue roller from puncture biopsy. Fixation of the material examined can be critical, too, and, therefore, we recommend as standard fixation procedure the use of buffered formalin and determining the fixation duration for 24 hours as maximum. It would be more suitable to use samples after prostatectomy in order to provide sufficient quantity of material.

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