Urol Res (1988) 16:341–344

Urological Research

Original Articles

c-myc Oncoprotein Levels in Bladder Cancer

J. R. W. Masters[1], S. G. Vesey[1], C. F. Munn[2], G. I. Evan[3], and J. V. Watson[2]

[1]Institute of Urology, St. Paul's Hospital, London and
[2]MRC Clinical Oncology Unit and [3]Ludwig Institute for Cancer Research, The Medical School, Cambridge, United Kingdom

Accepted: April 20, 1988

Summary. The protein coded by the oncogene c-myc, $p62^{c\text{-}myc}$, was measured using monoclonal antibodies and flow cytometry in nuclei derived from paraffin-wax sections of transitional cell carcinomas of the human bladder. Superficial disease (stages pTa and pT1) which did not recur within 5 years of diagnosis had significantly higher oncoprotein levels than those which did recur or were muscle-invasive (stage pT2 or greater) at presentation ($P < 0.01$). These preliminary findings indicate that oncoprotein levels might have prognostic significance for bladder cancer.

Key words: $p62^{c\text{-}myc}$ – Flow cytometry – Bladder cancer

Introduction

Superficial bladder tumours (stages pTa and pT1) account for between 75 and 85% of newly-diagnosed cases of transitional cell carcinoma. Between 40–80% of these tumours recur, usually within a year of presentation, and approximately 10% will develop more invasive disease (stage pT2 or greater). It is not possible to predict accurately which patients are at risk, although factors such as tumour stage, grade and multiplicity are of prognostic significance [8]. Thus, more specific correlates with prognosis could enable patient management to be planned more effectively.

Oncogenes are associated with fundamental cellular processes such as proliferation and neoplastic transformation. Recent studies indicate that two oncogenes, *onc*-D and c-*fgv*, encode the cytoskeletal elements tropomycin and actin, implicating cytoskeletal alterations in the process of invasion and metastasis [1, 16]. Expression of the c-*myc* gene is associated with changes in the cell-cycle from a non-proliferative to a stimulated state [7, 9]. The protein encoded by c-*myc*, $p62^{c\text{-}myc}$, is associated with the nucleus [2] and may be actively involved in DNA synthesis [13].

Monoclonal antibodies to $p62^{c\text{-}myc}$ have been raised [3]. These were used to localize this protein in tissue sections using immunocytochemical techniques [10, 12] and to measure quantitatively $p62^{c\text{-}myc}$ in nuclei extracted from stored paraffin-wax blocks [4, 6, 17, 18]. In testicular cancer levels of the protein were elevated and associated with the degree of differentiation and prognosis [18]. In uterine cervix, normal tissue exhibited higher protein levels than did carcinoma [6], while in contrast colonic carcinomas and severe dysplasia in ulcerative colitis showed elevated levels compared with normal mucosa [4, 11]. The purpose of this study was to obtain preliminary data to determine any association between $p62^{c\text{-}myc}$ levels and the development of recurrence or muscle invasion in bladder cancer.

Patients, Materials and Methods

Tissue from 39 patients was included in this study. The criteria for inclusion were the availability of sufficient paraffin-wax embedded biopsy of the primary tumour and complete follow-up data for a minimum of 5 years. The patients were divided into four groups as follows:

(a) Primary superficial transitional cell carcinoma (TCC) which did not recur within 5 years of diagnosis, during which period regular check cystoscopies were performed (Group SNR, 7 patients: 4Pa, 3P1).

(b) Primary superficial TCC which recurred as superficial TCC within 5 years of diagnosis (Group SSR, 15 patients: 6Pa, 9P1).

(c) Primary superficial TCC which recurred as invasive disease within 5 years of diagnosis (Group SI, 7 patients: 1Pa, 6P1).

(d) Primary invasive TCC (Group INV, 10 patients: 5P2, 5P3).

Sections 40 μm in thickness were cut from each block, dewaxed, rehydrated and then partially digested at 37 °C in 20 μg/ml pepsin (Sigma Ltd) in hydrochloric acid (pH 1.9) for 45 min, as described previously [17]. This is a modified version of the method of Hedley and colleagues [5], which releases nuclei by cytoplasmic digestion. The suspension containing nuclei was filtered through a 35 μm nylon mesh to remove large clumps and debris and was resuspended in 6 ml phosphate buffered saline (PBS) at pH 7.4. Aliquots of 1 ml

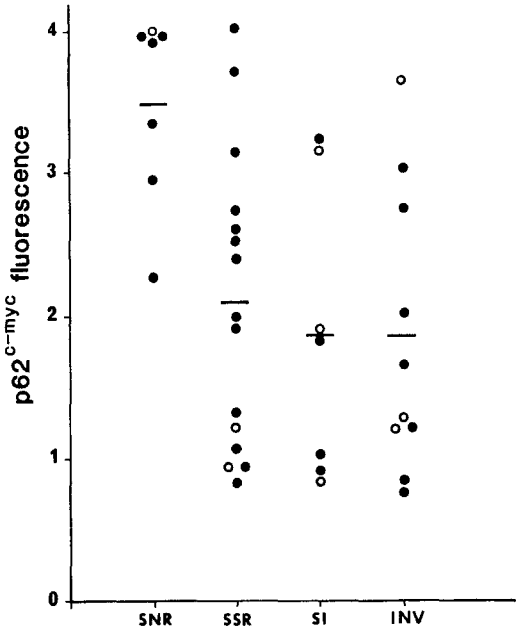

Fig. 1. Summary of $p62^{c\text{-}myc}$ levels. The median of the oncoprotein distribution associated with the diploid (solid symbols) or aneuploid (open symbols) components where present are shown for each disease category. The horizontal bars represent the means of the groups

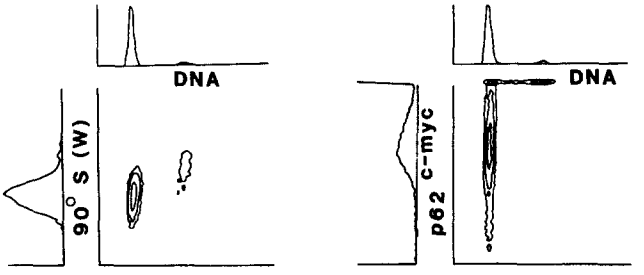

Fig. 2. The $p62^{c\text{-}myc}$/DNA assay for a superficial lesion which did not recur within 5 years of resection (SNR group). The left panel shows 90° light scatter (pulse width, proportional to diameter) on the ordinate plotted against DNA on the abscissa as a contour map. The G1 diploid peak is in channel 200 and a small G1 + M component is apparent. $p62^{c\text{-}myc}$ plotted against DNA is shown in the right hand panel

were placed in six Eppendorf tubes, centrifuged at 200 G for 5 min and the supernatants were removed.

Two anti-$p62^{c\text{-}myc}$ antibodies were used, MYC1-6E10 and MYC-CT14, and full details of their production are described elsewhere [3]. Two pellets were resuspended in 10 µl of the MYC1-6E10 antibody at dilutions of 1:10 and 1:31.6 and two in the same dilutions of the MYC-CT14 antibody. Following 45 min incubation at room temperature the samples were washed by adding 0.5 ml PBS, centrifuged at 200 G for 5 min and the supernatants were removed. The four samples plus one of the controls were then incubated with 10 µl fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin diluted 1:20 (Dako Ltd, Denmark). After 60 min 0.5 ml PBS was added to dilute the antibody and wash the samples, which were then centrifuged. The supernatants were removed and all samples resuspended in 0.5 ml of a solution containing the DNA fluorochrome propidium iodide, PI (Calbiochem Ltd), at a concentration of 50 µl/ml. Thus, one control contained nuclei stained with PI and fluorescinated antibody (fluorescence control) and the remaining 4 samples were stained with PI and two dilutions of both MYC1-6E10 and MYC-CT14 plus fluorescinated antibody.

The nuclei were analysed blind for DNA and $p62^{c\text{-}myc}$ simultaneously in the Cambridge MRC custom built dual laser flow cytometer [14], which incorporates a modified flow chamber to increase light collection efficiency [15]. The Innova 70-5 argon ion laser (Coherent, Palo Alto, CA) was tuned to the 488 nm line at a light power of 200 mW, which excites red fluorescence from the PI/DNA complex and green fluorescence from the fluorescein tagged oncoprotein. The green and red signals were separated by a 580 nm dichroic mirror (Zeiss Ltd) and the respective photodetectors were additionally guarded by a 515–560 nm band pass filter (green) and a 630 nm long pass filter (red). Forward and 90° light scatter were also collected. The instrument was calibrated so that a known G1 diploid DNA peak was recorded in channel 200 on the DNA (red) axis and at about channel 50 on the $p62^{c\text{-}myc}$ (green) axis. The signal in the green channel from the PI only control was due to red fluorescence breaking through the filters at the high voltages used on the green photomultiplier. This was an advantage as the instrument could be set up identically for each run by using the known diploid DNA peak as a standard for both channels.

Antibody specificity controls have been described previously [6, 9, 15, 16]. Briefly, three monoclonal antibodies which do not recognise $p62^{c\text{-}myc}$ or nuclear proteins and X-63 myeloma protein gave no signal above background and specific fluorescence was blocked by preincubation of MYC1-6E10 with the peptide used as the immunogen. Binding of the MYC1-6E10 antibody was not blocked with the peptide corresponding to the carboxy terminus of the c-myc protein. MYC-CT14 binding was blocked by the 32 amino acid carboxy terminus peptide but not with that used to produce the MYC1-6E10 antibody (residues 171–188).

The data were computer-collected and analysed to exclude clumps and debris. The median of $p62^{c\text{-}myc}$ fluorescence distribution associated with the diploid, triploid and tetraploid regions was calculated as described previously [17].

Results

The results obtained with the antibody MYC1-6E10 are shown in Fig. 1, where the median values of the $p62^{c\text{-}myc}$ fluorescence distributions are plotted for each disease category. The values given are those associated with the G1 diploid peak (closed circles, 30 patients) or with an aneuploid component if this was present (open circles, 9 patients). The superficial lesions showing no recurrence within 5 years (SNR) had significantly higher $p62^{c\text{-}myc}$ values than the other three groups ($P < 0.01$ for all comparisons, Mann-Whitney "U"-test) but there was no difference between the SSR, SI and INV groups.

Results with the MYC-CT14 antibody were qualitatively similar to those with MYC1-6E10. However, the signals with MYC-CT14 were four fold lower than those with MYC1-6E10, with a mean ratio of 3.95 and a standard error of 0.295. Nevertheless, in spite of the lower sensitivity with the MYC-CT14 antibody, the SNR group exhibited elevated $p62^{c\text{-}myc}$ fluorescence levels (mean = 847) compared with the other three groups (combined mean = 630), giving a statistically significant difference ($z = 1.9$, $P < 0.03$, Mann-Whitney "U"-test). Furthermore, the mean MYC1-6E10:MYC-CT14 ratio was 4.56 for the SNR group compared with 3.54 in

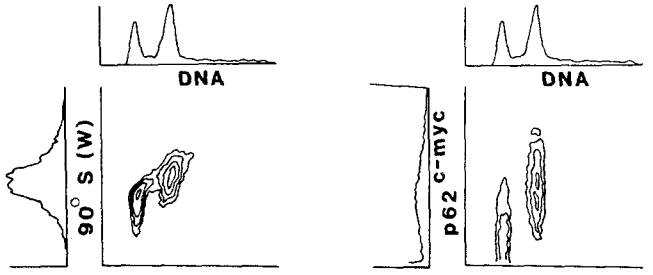

Fig. 3. Data comparable with figure 2 from a muscle-invasive tumour (INV group) which exhibits an aneuploid peak. 90° light scatter (pulse width) is plotted against DNA in the left panel and $p62^{c\text{-}myc}$ versus DNA is shown on the right. The diploid DNA component (channel 200) and the aneuploid peak are clearly discernible as are both the light scatter and $p62^{c\text{-}myc}$ distributions associated with these populations with different DNA content

the remaining groups combined. The Mann-Whitney "U"-test comparing the ranking of the ratios in these two groups for a large sample was significantly different ($z = 1.68$, $P < 0.05$).

Two examples of the simultaneous DNA/$p62^{c\text{-}myc}$ assay, one from a diploid and one from an aneuploid lesion, are given. The data in Fig. 2 were obtained from a superficial lesion which did not recur within 5 years of resection (SNR group). The left panel shows 90° light scatter (pulse width, which is proportional to diameter) on the ordinate plotted against DNA on the abscissa as a contour map. The G1 diploid peak is in channel 200 and a small G1 + M component is apparent. The right panel shows $P62^{c\text{-}myc}$ plotted against DNA for this same sample. Figure 3 shows comparable data from an invasive tumour (INV group) which exhibits an aneuploid peak. 90° light scatter (pulse width) is plotted against DNA in the left panel and $p62^{c\text{-}myc}$ versus DNA is shown in the right panel. The diploid component (channel 200) and the aneuploid peak are clearly discernible as are both the light scatter and $p62^{c\text{-}myc}$ distributions associated with these populations with different DNA content.

Discussion

The significant finding in this investigation was that transitional cell carcinoma of the bladder which did not recur within 5 years of resection had higher $p62^{c\text{-}myc}$ oncoprotein levels than lesions invading muscle and those recurring within this interval. In our studies on uterine cervix the normal mucosa also exhibited higher $p62^{c\text{-}myc}$ levels than carcinomas [6], and well-differentiated colonic carcinomas [11] and teratomas [18] had higher levels than poorly differentiated tumours. Because c-myc is associated with cell division, the opposite result might have been expected. Explanations for this apparent anomaly include posttranslational modification or c-myc gene mutation in more aggressive cells, increased turnover of the protein which is known to have a short half-life in stimulated cells [9] or an increase in the susceptibility of the protein to proteolysis in the more aggressive cells during preparation for the assay.

The antibodies used in this study were raised to peptide sequences corresponding to the normal protein and the MYC1-6E10 antibody gave a four-fold higher signal compared with MYC-CT14. In the uterine cervix studies we postulated that similar results could have been due to different protein binding constants for MYC1-6E10 in normal and malignant cells due to an altered protein structure, as the maximum signal occurred at different antibody concentrations in the different cell types [6]. In this study all specimens showed the signal at the same concentration with both antibodies (1:10), but the MYC1-6E10:MYC-CT14 ratio was significantly different ($P < 0.05$) comparing the group which did not recur with the rest. However, the significance of this difference is small and insufficient to suggest that there could be changes in binding constants for the respective antibodies in the lesions with the more aggressive phenotype. It is possible that the normal function of this protein could be bypassed in the carcinogenic process in some tissues, leading to lower nuclear content in carcinomas arising from those tissues. Further studies will be required using both fresh and fixed tissue to test these hypotheses.

Although we do not yet understand the function of $p62^{c\text{-}myc}$ the findings have clinical relevance as they provide preliminary evidence that at least one oncoprotein has prognostic significance in bladder cancer. More cases will be needed to relate oncoprotein levels to stage and grade, in order to determine whether these measurements provide distinct and additional prognostic information. The possibility exists that measurements of the levels of a panel of oncoproteins will provide the clinician with sufficient information to accurately predict the outcome of the disease.

References

1. Bishop JM (1987) The molecular genetics of cancer. Science 235:305–311
2. Evan GI, Hancock DC (1985) Studies on the interaction of the human *c-myc* protein with cell nuclei: $p62^{c\text{-}myc}$ as a member of a discrete subset of nuclear proteins. Cell 43:253–261
3. Evan G, Lewis GK, Ramsay G, Bishop JM (1985) Isolation of monoclonal antibodies specific for human and mouse proto-oncogene products. Mol Cell Biol 5:3610–3616
4. Forgacs IC, Sunderesan V, Evan G, Wight DGD, Neale G, Hunter JO, Watson JV (1986) Abnormal expression of *c-myc* oncogene product in dysplasia and neoplasia associated with ulcerative colitis. Gut 27:A1285
5. Hedley DW, Friedlander MI, Taylor IW, Rugg CA, Musgrove EA (1983) Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333–1335
6. Hendy-Ibbs P, Cox H, Evan GI, Watson JV (1987) Flow cytometric quantitation of DNA and *c-myc* oncoprotein in archival biopsies of uterine cervix neoplasia. Br J Cancer 55:215–282
7. Kelly K, Cochran BH, Stiles CD, Leder P (1984) The regulation of *c-myc* by growth signals. Curr Top Microbiol Immunol 113:117–126

8. Lum BL (1983) Intravesical chemotherapy of superficial bladder cancer. Recent Results Cancer Res 85:3–36
9. Rabbitts PH, Watson JV, Lamond A, Fischer W, Forester A, Stinton MA, Evan GI, Atherton E, Sheppard RC, Rabbitts TH (1985) Metabolism of c-*myc* gene products: c-*myc* mRNA and protein expression in the cell cycle. EMBO J 4:2009–2015
10. Sikora K, Evan G, Stewart J, Watson JV (1985) Detection of the c-*myc* oncogene product in testicular cancer. Br J Cancer 52:171–176
11. Sikora K, Chan S, Evan G, Gabra H, Markham N, Stewart J, Watson JV (1987) c-*myc* oncogene expression in colorectal cancer. Cancer 59:1289–1295
12. Stewart J, Evan GI, Watson JV, Sikora KE (1986) Detection of the c-*myc* oncogene product in colonic polyps and carcinomas. Br J Cancer 53:1–6
13. Studzinski GP, Brulei ZS, Feldman SC, Watt RA (1986) Participation of c-*myc* protein in DNA synthesis of human cells. Science 234:467–470
14. Watson JV (1980) Enzyme kinetic studies in cell populations using fluorogenic substrates and flow cytometric techniques. Cytometry 1:143–151
15. Watson JV (1985) A method for improving light collection by 600% from square cross section flow cytometry chambers. Br J Cancer 51:433–435
16. Watson JV (1986) Oncogenes, cancer and analytical cytology. Cytometry 7:400–410
17. Watson JV, Sikora KE, Evan GI. (1985) A simultaneous flow cytometric assay for c-*myc* oncoprotein and cellular DNA in nuclei from paraffin embedded material. J Immunol Methods 83:179–192
18. Watson JV, Stewart J, Ritson A, Evan GI, Sikora EK (1986) The clinical significance of flow cytometric c-*myc* oncoprotein quantitation in testicular cancer. Br J Cancer 53:331–337

J. Masters, PhD
Institute of Urology
St. Paul's Hospital
24 Endell Street
London WC2H 9AE
United Kingdom