# Mechanical properties of 67NR tumours are broadly independent of the immune status of the host mouse

Catherine Taylor Nordgård[1], Shalini V. Rao[2], Morten J. Dille[1], Tonje S. Steigedal[2], and Kurt I. Draget[1]

[1] Department of Biotechnology and Food Science, Norwegian University of Science and Technology NTNU, Trondheim, Norway
[2] Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology NTNU, Trondheim, Norway

## ABSTRACT

Tumour stiffness is increasingly seen as a clinically relevant parameter, with increased stiffness being linked to poorer prognosis, but it is also one that is not inherently easy to measure either *in vivo* or *ex vivo*. We have established a testing protocol for fresh ex vivo tumour tissue from tumour bearing mice using a Stable Micro Systems texture analyser fitted with a 2mm diameter cylindrical probe. The data demonstrate that, in this tumour model, changes in tumour growth time alter the tumour stiffness, but the presence or absence of T-cells does not significantly alter tumour stiffness. However, there may be more substantial differences if a more immunogenic tumour model was employed.

## INTRODUCTION

Breast cancer is often first identified after discovery of a 'lump' upon breast palpation, something which is directly linked to the increased stiffness of tumour tissue compared to normal breast tissue. Not only is tumour tissue stiffer than normal tissue but stiffer, fibrotic tumour tissue is correlated with poorer clinical outcomes when compared to less stiff or fibrotic tumours[1-4].

Reported tumour stiffness measurements are predominantly based on imaging not mechanical or rheological measurements[1, 5]. Imaging studies have a clear clinical advantage in that they may be performed *in situ*, however there is also evidence that imaging elastography and tissue compression may not correlate in the same way with histopathological findings relating to fibrosis[6]. This suggests there is a role for mechanical analysis of tumour tissue.

As part of a larger study we had available freshly excised 67NR tumour tissue from BALB/c and Nude mice. The 67NR cell line is a non-metastatic cell line derived from a spontaneous tumour growing in a Balb/cfC3H mouse[7] and 67NR tumours can be established in both immunocompetent (BALB/c) and immunocompromised (Nude) mice. Given the potential role of T-cells (which Nude mice lack) in tumour control[8] it is of interest to investigate whether there are differences in stiffness between 67NR tumour grown in BALB/c and Nude mice.

Mechanical characterisation (texture analysis) of solid and semi-solid (soft) complex materials is widespread in the food industry[9], which has driven much of the instrumental development in this field but also in use in, for example, the pharmaceutical field[10]. In this investigation we utilise a Stable Micro Systems texture analyser to assess the mechanical properties of freshly excised 67NR tumour tissue.

METHODS

*In vivo* methods - cells

67NR cells were sub cultured and maintained into a continuous cell culture in DMEM (GIBCO) supplied with 10% FBS, 250 µg/ml amphotericin B (Sigma-Aldrich), 100 µg/ml gentamycin (Invitrogen).

*In vivo* methods – animals

This study was approved by the Animal Welfare Committee at St. Olavs Hospital in Trondheim. Controls were age matched littermates. All mice were housed in wire-top cages with aspen wool chip bedding. Room temperature was 24±1°C with a relative humidity of 45-50% and a 12-H light/dark cycle. Mouse Diet and tap water were provided ad libitum.

*In vivo* methods – treatment and analysis

The 67NR is derived from a spontaneous tumor in a Balb/cfC3H mouse. 67NR cells were procured from Richard O. Hynes lab. They were taken up a week before the injection liquid nitrogen cell stock and maintained in a continuous subculture in DMEM. $20x10^6$ cells per mL of cell suspension was prepared in PBS and kept at $4^oC$ on ice throughout the cell instillation procedure. 20 uL of this cell suspension that contained $4x10^5$ cells in total was installed by orthotopic injection in the left tertiary fat pad of 7-8 week old female BALb/c mice which were anesthetized with Isoflurane.

The tumor volumes were measured, by electronic vernier calipers 3 times a week. The body weight was measured twice a week throughout the experiment. The tumour volumes were calculated using the formula of the volume of a prolate,

$$V_{prolate} = \frac{4}{3}\pi a^2 b$$

$a$ is the tumor width and $b$ is the tumor length[16].

After 17 or 25 days the mice were sacrificed by cervical dislocation. Tumours were excised and bisected with one half of the fresh tumour tissue used to measure mechanical stiffness.

Mechanical examination of tumour tissue

Tumours were placed cut side down on a piece of tissue (to avoid slippage) on the stage of Stable Micro Systems TA.XT plus texture analyser fitted with a 2mm cylindrical probe. The probe was chosen to minimise the influence of irregular tumour shape and size on the data generated. In the absence of obvious inhomogeneities in the tumour surface the tumour was centred under the probe. If necessary the placement was adjusted to avoid penetration in areas of obvious inhomogeneity. Tumours were tested in compression mode with a single compression to 75% strain. Results were plotted as force strain curves.

RESULTS

Tumour growth is independent of immune status of mouse and all three groups show similar growth to 17 days (Figure 1). The growth curves are similar to published data from this tumour model[11].

Force distance curves for BALB/c 25 days show relatively low resistance to compression and force strain curves are broadly linear (Figure 2).

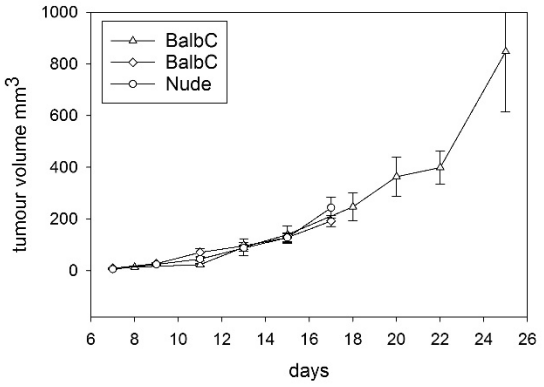


Figure 1. Tumour volume for orthotopic 67NR tumours implanted in BALB/c mice over 17 and 25 days, and in Nude mice over 17 days.

For both BALB/c and Nude mice tumours removed at 17 days the curves showed profiles typical of elastic hydrogels [12, 13] with the gradient of the force strain curve increasing with increasing strain until penetration occurred resulting in a drop in force (Figure 2). In some instances the force begins to rise again after a penetration event (Figure 2) and in these cases dissection of the tumour after penetration often revealed areas of gross necrosis which presumably offer much less resistance to penetration than the surrounding tumour tissue.

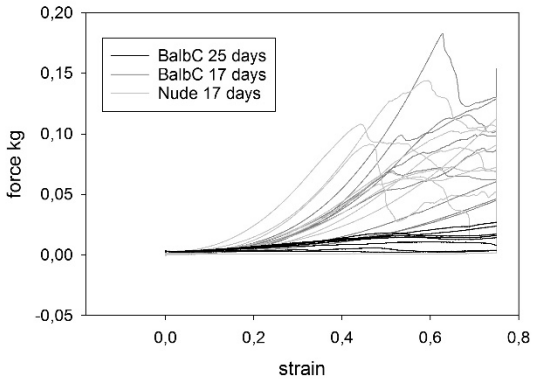


Figure 2. Force distance curves for compression with 2mm cylindrical probe to 75% strain for individual 67NR tumours grown in BALB/c mice for 25 days, BALB/c mice for 17 days and in Nude mice for 17 days.

Penetration events are not detected below 40% strain for any sample (Figure 2). The force at 40% strain is significantly higher for the tumours in BALB/c mice grown for 17 days than the tumours in BALB/c mice grown for 25 days (two-tailed P value = 0.016 t-test, SigmaPlot) (Figure 3). The force at 40% strain has greatest variability in the tumours from the Nude mice allowed to grow for 17 days. Differences between the Nude and BALB/c mouse tumours at 17 days do not reach statistical significance (Figure 3).

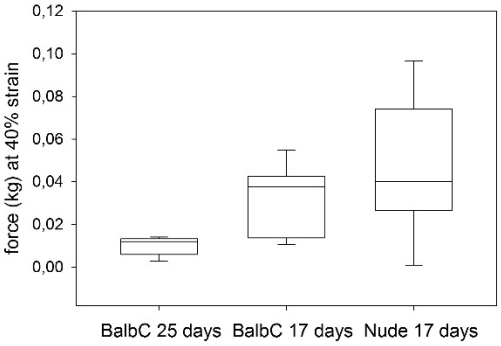


Figure 3. Box plot showing the median, 10th, 25th, 95th and 90th percentile force (in kg) at 40% strain for 67NR tumours grown in BALB/c mice for 25 days, BALB/c mice for 17 days and Nude mice for 17 days.

Tumour density was estimated after tumour excision by measuring the weight of the tumour and estimating the volume of the tumour based on axis measurements. The densities of the tumours within a group are somewhat variable (Figure 4), which may in part reflect inherent inaccuracies in volume calculations. The tumours grown in BALB/c mice for 25 days were significantly less dense than the tumours grown in BALB/c mice for 17 days (two-tailed P value = 0.00096, t-test, SigmaPlot), whereas there was no significant difference between the densities of the tumours grown in BALB/c and Nude mice for 17 days.

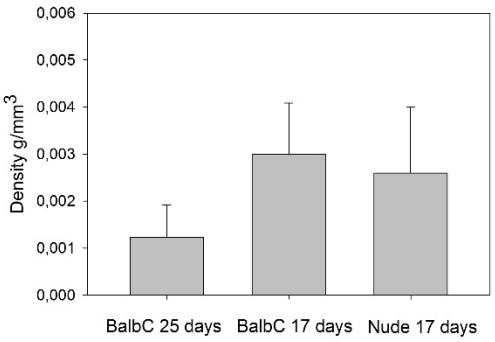


Figure 4. tumour density in g/mm$^3$ (mean + S.D.) assessed after tumour excision for 67NR tumours grown in BALB/c mice for 25 days, BALB/c mice for 17 days and Nude mice for 17 days.

Tumour density is known to be variable [14] but is often assumed as constant in *in vivo* studies [15].

## DISCUSSION

The data presented here show the 67NR tumour growth curves *in vivo* are reproducible, with no significant differences between the groups at the same time point (Figure 1), suggesting in this model tumour growth is not inhibited by the presence of T-cells. This fits with the fact that this cell line is derived from a tumour that arose spontaneously in a BALB/c mouse with an intact immune system including T-cells[7], and with histological data (not shown) that shows minimal T-cell invasion of the tumour tissue suggesting 67NR is not a particularly immunogenic tumour model.

There are however clear differences in the force/strain curves of the longest growing tumours vs the shorter growing tumours in BALB/c mice (figures 2 & 3), indicating the texture analysis method is able to distinguish changes in tumour stiffness. In the BALB/c mouse, increased growth time for 67NR tumours led to accelerated tumour growth (Figure 1), softer, less typically elastic tumours (Figures 2 & 3) and a reduction in tumour density (Figure 4). These factors may be interconnected. One possible consequence of rapid growth may be a functional reduction in the concentration of macromolecular components, such as extracellular matrix molecules due to biosynthetic constraints[17,18]. As these components contribute to tissue stiffness a reduction in their functional concentration may result in a reduction in tissue stiffness. It is also possible that such functional concentration changes also result in a reduction in tissue density that might affect cellular response[19,20].

The compression studies of tumour tissue show relatively high variability but this is to be expected given its biological nature of the test material (tumour) and the presence of in homogeneities both within the tumour tissue architecture and as a result of necrotic processes. Despite the variability we were able to detect clear differences in mechanical properties depending on the growing time of the tumours (Figures 2 & 3), which may be related to tumour density changes (Figure 4).

Variability in mechanical properties is greatest in the Nude mice, this may reflect subtle changes in the way the animal responds to the tumour as a result of the lack of T-cells however broadly speaking the immune status of the host mouse had no significant effects on either the tumour growth or the mechanical properties of the tumour tissue.

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C. T. Nordgård and S. V. Rao have contributed equally to the work.

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