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ARAŞTIRMA MAKALESİ/**RESEARCH ARTICLE**

DNA INDEX AND CELL CYCLE ANALYSES BY FLOW CYTOMETRY TECHNIQUES OF NORMAL AND ABNORMAL CELLS OF BREAST TISSUES

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ABSTRACT

This study was carried out in the Department of Pathology, Faculty of Medicine at Trakya University and Department of Biology, Faculty of Art and Sciences at Trakya University in 1991-1995. In the study, normal and abnormal tissue samples obtained from the patients, whose were suspected for cancer were wed. Initially, pathological characters of the tissues were identified. Then these tissues were analysed by using Flow cytometry technique. Also, Flow cytometry technique was used in order to show DNA index of the cells. Also, it can be used to show the changes in the cell cycle phases, which related to produce cancerous tissue. Results shoved statistically significant differences in DNA index analyses and in cell cycle values. However, it can be said that this method alone, cannot be reliable enough in clinical diagnosis. Because some values were deviated from the mean values. Therefore in order to increase the reliability of the method, a standardization, which may be supported by some other techniques, is required. Moreover, the role of G_0 cells on the ratio differences of G_1+G_0 cells values in terms of normal and abnormal cells should be determined.

Key Words: Flow cytometry, DNA index, Cell cycle, Breast cancer.

NORMAL VE ANORMAL KARAKTERLİ MEME DOKULARINDA FLOW SİTOMETRİ TEKNİĞİ İLE DNA İNDEKSİ VE HÜCRE SİKLUSU ANALİZLERİ

ÖZ

Bu araştırma 1991-1995 yılları arasında Trakya Üniversitesi Tıp Fakültesi Patoloji Anabilim Dalı ve Trakya Üniversitesi Fen-Edebiyat Fakültesi Biyoloji Bölümünde yürütülmüştür. Çalışmada, meme kanseri şüpheli hastalardan alınan normal karakterli dokular kullanılmıştır. Dokuların karakterleri patolojik inceleme ile belirlenmiştir. Daha sonra bu örnekler Flow sitometri tekniği kullanılarak değerlendirilmiştir. Çalışmada ayrıca kanser oluşumunda kanserli dokuyu oluşturan hücrelerdeki DNA indeksi ve hücre siklusunun fazlarında oluşan değişimlerin Flow sitometri yöntemi ile incelenmesi amaçlanmıştır. Çalışma sonucunda, DNA indeksi değerleri açısından ve hücre siklusu analizlerinde elde edilen ortalamalarda istatistiksel olarak anlamlı sonuçlar elde edilmiştir. Ancak, ortalamalara uymayan değerlere sahip örneklerinde bulunması nedeniyle bu yöntemin klinik tanıda tek başına kullanımının henüz yeterli derecede güvenilir olmadığı kanısına varılmıştır. Yöntemin güvenilirliğinin arttırılabilmesi için Flow sitometri çalışmalarının değerlendirilmesinde bir standartizasyonun sağlanması ve ayrıca diğer yöntemler ile pekiştirilmesi gerekliliği görülmüştür Ayrıca normal ve anormal gruplar arasında G_1+G_0 hücre oranı değişiminde G_0 hücrelerinin rolünün saptanması gerektiği kanısına varılmıştır.

Anahtar Kelimeler: Flow sitometri, DNA indeksi, Hücre siklusu, Meme kanseri.

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1. INTRODUCTION

Despite of the numerous researches, the cancer still remains as a threading disease for the man. The main reasons for its danger are threefold: 1- The large number and diversity of possible causes, 2- The difficulties of eliminating them 3- The challenges confronted throughout the period of hospitalisation including those of the diagnosis and treatment (Patıroğlu, 1994). The research in the field hitherto has revealed that in many cases, early diagnosis is possible and thus the illness at this stage is likely to be treated. However, if the illness is advanced the treatments may not be very promising. The illness is being diagnosed only when it manifests in such a way that its pathological effects can be well defined.

After this stage, the treatment becomes difficult. The histopathological or cytological method, however, brings about the disadvantage of giving several different results upon the examination of the same case due to misjudgements when deriving the specimen from the patient. This method is often supported by the methods of ultrasonography, immunocytochemistry, radiology, and flow cytometry (FCM) at the stage of the diagnosis. In the FCM, it is possible to make diagnoses depending on the DNA quantity distribution in the cells and cell-cycle analyses, which are integral to the method itself (Eskelinen et al., 1989a; Hedley et al., 1987; Keren, 1994; Safalı et al., 1992). In fact, when examining a tissue specimen, a rapid, practicable and repeatable method is generally required. This makes it is possible to look into the specimen at the cellular level, which clearly asses .. ..hat extent the specimen in question is formed of the abnormal cell population. Thus it provides the capability to make estimations in numbers.

The FCM is a rapid and repeatable method and is possible to apply to fresh tissues and fixed tissues (Harve et al., 1993; PhilipMcCoy, 1994; O'hara et al., 1984). It has been reported that the FCM has additional reliability because of its capability to work on a numerous collection of the cells at the same time. The FCM recognises the variation observed in the cell cycle as well as estimating the quantity of DNA in the cell (Philip McCoy, 1994; Coon et al., 1987; Merkel and McGuire, 1990). Owing to the ability to observe the cell cycle changes, the FCM determines the increase of the cells at the S-phase with diploid character. Unlike the fresh tissues, the FCM is repeatable to the tissues fixed in paraffin blocks, which has the further advantage of not mixing the abnormal tissues and to the normal ones as usually observed in fresh tissue biopsy (Harvey et al., 1993). The FCM method can be considered additional to histological and cytological methods as well as with its increased reliability. In fact, the errata due to wrong diagnosis sometimes may decrease the reliability of cytolological methods.

Our main purpose for this study was to assess the extent the FCM differentiation in terms of the cell kinetics and to see if the FCM is applicable and beneficial in the clinical research, considering it is a method which could facilitate the arduous process of pathological evaluation in the clinical research.

2. MATERIAL AND METHOD

The study materials were obtained from the patients suspected for breast cancer in the Pathology Department of Faculty of Medicine at Trakya University during 1991-1995. The specimens were prepared with paraffin block and the stained sections with Hematoxilen-Eozin were used for pathological identifications. A pathologist performed identifications. Ten sections (30-40 µ) were used for specimen. These sections were prepared for FCM treatments. Then, each one was taken into preparation and was tested through the FCM (O'hara et al., 1984; Nicholson, 1989). The samples were placed in the test-tubes for the preparation, implementing the required measurements within the necessary duration (deparaffinisation in Xylen for 15-20 minutes at 20°C, bathing in alcohol, transferring through low-alcohol-density series, keeping in physiological liquid for 24 hours, keeping phosphate buffer). The samples were then taken into detergent (0.6 % labelled Non IDET, SIGMA) and the DNA within was marked by Propidium Iodine (labelled SIGMA P-4170 Lot 35h3667). A FCM device used in the present study was calibrated with the admixture of the DNA-QC solution (Cat.No. 95-0023, BECTON-DICKINSON) and the DNA Cycletest-DNA reagent kit (Cat.No.95-2000, BECTON-DICKINSON), choosing both solutions from the same brand with the FCM device. The solution containing the cells is placed in the tube integral total device, and the necessary measurements were performed. These data are shown quantitatively as a histogram (Figure 1). During the evaluation of the results, the first criteria was the use of the DNA index.

While the DNA index is obtained, the G_1+G_0 peak has been formed in the histogram as 200 (the unit referring to the amount of DNA). This data refers to (2n) diploid DNA quantity. In our case, the level of G_1+G_0 peak was derived from the observed group of cells Thus, our G_1+G_0 peak is DNA index (DI). The DI=1 cells are diploid, while the cells whose DI has been measured below or above 1 are aneuploid. For example, those between 1.05-2.0 are aneuploidş, and those above 2 are tetraploid. Since DI is being considered to correspond to the number of chromosomes within the cells, 2n refers to the diploid, 3n to the triploid and 4n to the tetraploid cells respectively (Overton, 1994; Shapiro, 1989; Batsakis et.al., 1993; Dowle et al., 1987; Throud et al.,1986; Kallioniemi et al., 1987; Baildam et al.,

1987; Yuan et al., 1992; Auer et al., 1980; Daidone et al., 1995).

Another criterion has been the change in the ratio of the cells within the cell cycles. Hence, it is possible to compare and to contrast both groups in terms of the changes in the ratios of G_1+G_0, S and G_2+M. The cells at G_1+G_0 phase are 2n (diploid), those at M and G_2 phase are 4n (tetraploid), and those at S phase exert a change ranging between 2n and 4n (Overton, 1994; Shapiro, 1989; Batsakis et al., 1993; Dowle et al., 1987; Kallioniemi et al., 1987; Yuan et al., 1992; Daidone et al., 1995; Vendrly, 1971). The statistically analyses were performed by using the Wilcoxon's Signed-Ranks Test (Hayran and Özdemir, 1995).

3. RESULTS

The values of the two groups were compared to reveal the statistical significance (of which the first containing the normal cells and the second, the abnormal cells) which was histopathological evaluated by a pathologist. A Wilcoxon's Signed-Ranks Test was performed to reveal the results. The results were as follows:

The DI normal group was 1.08, and its standard deviation was 0.0009, while the other, "tumour" group had a DI of 1.51 (Figure 2) with a standard deviation of 0.39 (Table 1). When the DI values of either groups were compared by means of Wilcoxon's Signed-Ranks Test with a standard deviation of 0.05, the result was significant ($P<0.001$). The mean of the tumour group DI indicates an apparent increase in comparison to that of the normal group.

The mean values of the G_1+G_0 in the normal group was 79.5, with a standard deviation of 11.4 while the other, "tumour" group had a G_1+G_0 mean of 58.3 (Figure 3) with a standard deviation of 23.3 (Table 2). The G_1+G_0 values of either groups were compared by means of Wilcoxon's Signed-Ranks Test with a standard deviation of 0.05, and the result obtained was significant ($P<0.001$). The mean G_1+G_0 values of tumour group indicates a marked decrease in comparison to that of the normal group.

The mean values of the normal group S was 16.7, and its standard deviation was 10.5 while the other, "tumour" group had an S mean of 25.0 (Figure 3) with a standard deviation of 20.0 (Table 3). Thus the result obtained was significant ($P<0.01$). The mean S value of tumour group indicates significant increase in comparison to that of the normal group.

The mean values of G_2+M in the normal group was 3.8, with a standard deviation of 3.5, while the other, "tumour" group had a G_2+M mean of 17.0 (Figure 3) with the standard deviation of 16.8 (Table 4). When the G_2+M values of either groups were compared by means of Wilcoxon's Signed-Ranks Test at a standard deviation of 0.05, the result was significant ($P<0.001$). The mean G_2+M of tumour group indicates significant difference from the normal group.

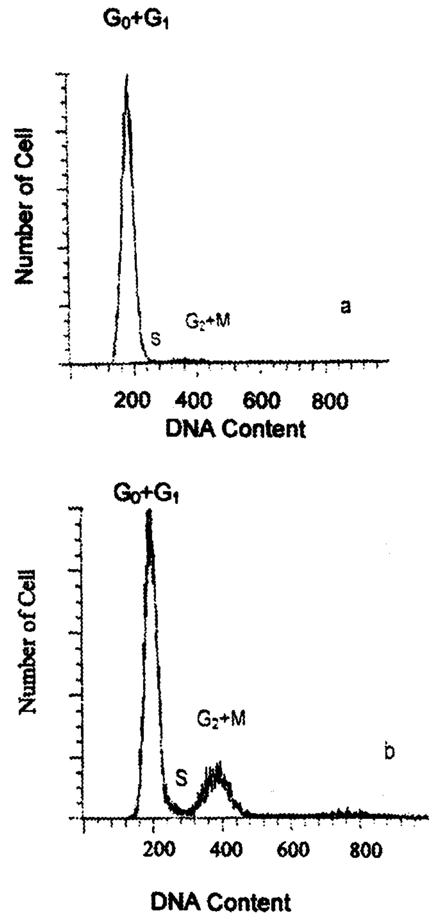

Figure 1. Examples The FCM Histogram's Of Two Groups: a) Normal Group, b) Abnormal Group (200=2n=2c DNA Content).

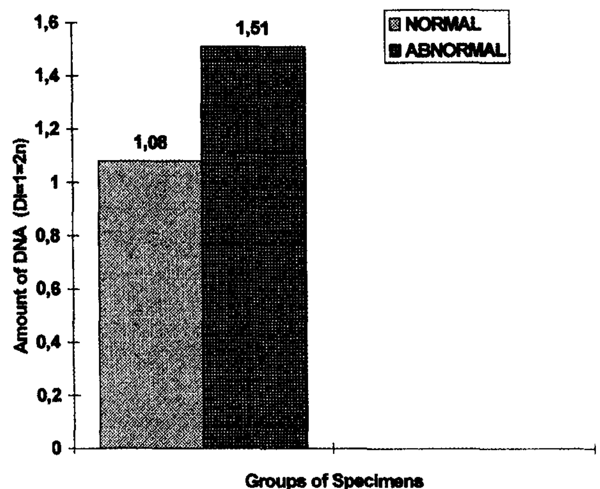

Figure 2. Histogram Of DI Of Normal and Abnormal Groups.

Table 1. DNA Index (DI) For Normal and Abnormal Groups (ten sections were used for each specimens).

Specimen No.	DI of normal cells	DI of abnormal cells	Specimen No.	DI of normal cells	DI of abnormal cells
1	1.21	2.00	11	1.15	1.80
2	1.00	1.20	12	1.26	1.24
3	1.00	1.67	13	1.00	1.82
4	1.17	1.26	14	1.00	1.48
5	1.21	1.53	15	1.00	1.99
6	1.14	2.00	16	1.18	2.01
7	1.00	1.00	17	1.00	1.00
8	1.11	1.05	18	1.00	2.04
9	1.00	1.55	19	1.00	1.00
10	1.22	1.19	---	---	---
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Table 2. Number and % G1+G0 Phases of Normal and Abnormal Groups (ten sections were used for each specimens).

Specimen No.	Total cells of normal specimens	Number of cells examined normal specimens	Number G_1+G_0 cells of	% G_1+G_0 of normal specimens specimens	Total cells of abnormal	Number of cells examined	Number G_1+G_0 cells of abnormal specimens	% G_1+G_0 of abnormal specimens
1	4234	1026	754	73.5	7429	6967	3669	52.2
2	9173	9013	8338	92.5	12544	3263	826	25.3
3	10704	10314	9071	87.9	19146	7618	5579	73.2
4	12455	11293	8315	73.6	15433	3686	1104	30.0
5	13390	9021	5658	62.7	13596	1618	292	18.0
6	4300	3552	2725	76.2	20705	15073	2889	19.2
7	17153	15559	13921	89.5	17304	15702	12739	81.1
8	12569	6617	4380	66.2	16530	6743	3072	45.6
9	15988	15820	15162	95.8	14225	3019	2421	80.2
10	17014	14361	11165	77.7	16143	11325	7959	70.3
11	12800	11028	8669	78.6	15864	9730	8388	85.2
12	15844	8597	6221	72.4	15221	3570	1677	47.0
13	18049	17155	15617	91.0	9762	5865	4115	70.2
14	4195	3596	3134	79.2	12948	6356	2275	34.8
15	17014	16082	12346	76.8	17254	4470	3154	70.5
16	15353	4833	2690	55.7	14792	13353	9635	72.2
17	11803	11761	11761	95.3	12503	11706	9992	85.4
18	13107	12968	11847	91.4	11665	4587	3617	78.9
19	19245	18356	13638	74.3	18703	17926	12942	69.2
Mean±SD	----------	----------	----------	79.5±11.4	----------	----------	----------	58.3±23.3

Table 3. Number And Percentage Of Normal And Abnormalcells In The S Phase (ten sections were used for each specimens).

Specimen No	Total cells of normal specimens	Number of cells examined	Number S cells of normal specimens	% S of normal specimens	Total cells of abnormal specimens	Number of cells examined	Number of cells of abnormal specimens	% S of abnormal specimens
1	4234	1026	253	24.7	7429	6967	1394	20.0
2	9173	9013	540	6.0	12544	3263	2057	63.0
3	10704	10314	1134	11.0	19146	7618	1461	19.2
4	12455	11293	2946	26.1	15433	3686	2341	63.5
5	13390	9021	3248	36.0	13596	1618	1061	65.6
6	4300	3552	784	22.1	20705	15073	2738	18.2
7	17153	15559	1054	6.8	17304	15702	2635	16.8
8	12569	6617	1801	27.2	16530	6743	2488	36.9
9	15988	15820	305	1.9	14225	3019	353	11.7
10	17014	14361	1983	13.8	16143	11325	2312	20.4
11	12800	11028	2114	19.2	15864	9730	383	3.9
12	15844	8597	2230	25.9	15221	3570	1623	45.5
13	18049	17155	1488	8.7	9762	5865	444	7.6
14	4195	3596	626	15.8	12948	6356	486	7.4
15	17014	16082	1999	12.4	17254	4470	942	21.1
16	15353	4833	1759	36.4	14792	13353	272	5.9
17	11803	11761	519	4.5	12503	11706	1627	13.9
18	13107	12968	532	4.1	11665	4587	726	15.8
19	19245	18356	2763	15.1	18703	17926	3204	16.9
Mean±SD	---------	--------	-----------	16.7±10.5	----------	----------	----------	25±20

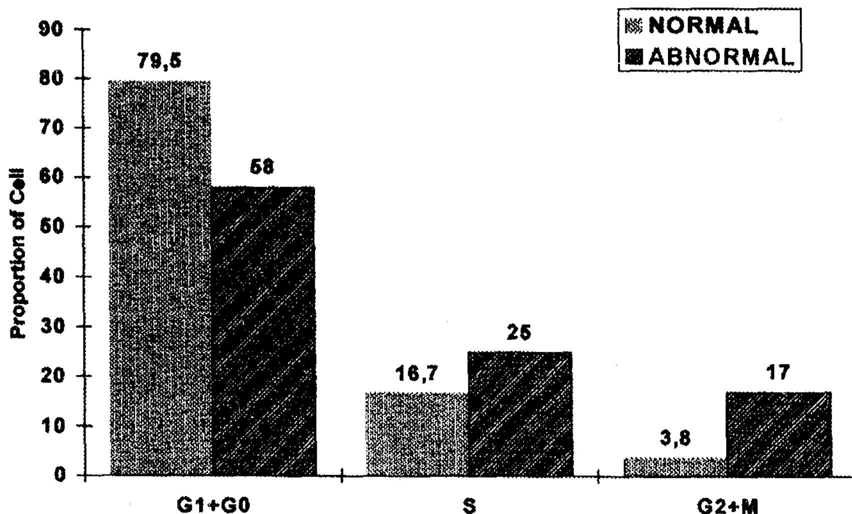

Figure 3. Histogram Of Cell Cycle Phases Of Normal And Abnormal Groups.

Table 4. Number and % G2+M Of Normal and Abnormal Groups (ten sections were used for each specimens).

Specimen No	Total cells of normal specimens	Number of cells examined	Number G2+M cells of normal specimens	% G2+M of normal specimens	Total cells of abnormal specimens	Number of cells examined	Number G2+M cells of abnormal specimens	% G2+M of abnormal specimens
1	4234	1026	19	1.9	7429	6967	1904	27.3
2	9173	9013	135	1.5	12544	3263	380	11.6
3	10704	10314	109	1.1	19146	7618	578	7.6
4	12455	11293	32	0.3	15433	3686	241	6.5
5	13390	9021	115	1.3	13596	1618	265	16.4
6	4300	3552	43	1.2	20705	15073	9446	62.7
7	17153	15559	584	3.8	17304	15702	328	2.1
8	12569	6617	436	6.6	16530	6743	1183	17.5
9	15988	15820	353	2.2	14225	3019	245	8.1
10	17014	14361	1213	8.4	16143	11325	1054	9.3
11	12800	11028	245	2.2	15864	9730	1009	10.4
12	15844	8597	146	1.7	15221	3570	270	7.6
13	18049	17155	50	0.3	9762	5865	1306	22.3
14	4195	3596	196	5.0	12948	6356	3375	57.8
15	17014	16082	1737	10.8	17254	4470	377	8.4
16	15353	4833	384	7.9	14792	13353	2926	21.9
17	11803	11761	29	0.2	12503	11706	87	0.7
18	13107	12968	589	4.5	11665	4587	244	5.3
19	19245	18356	1955	10.7	18703	17926	2500	13.9
Mean±SD	----------	----------	----------	3.8±3.5	----------	--------	----------	17±16.8

4. DISCUSSION

The DI values of malign breast tumour cells are considered as follows: those between 1.0-1.05 as diploid and those above 1.05 as aneuploid (Keren, 1994; Nicholson, 1989; Overton, 1994; Battakis et al., 1993; Kallioniemi et al., 1987; Auer et al., 1980). Our study results differ from all the previous study in that determining the base and peak values of the diploid, the DI mean of cell populations of normal character cells (from each patient) were sought and found as 1.0-1.08. The cell population DI mean of abnormal character cells were found as 1.51 (Figure 2). Since these results are significant, it is worth mentioning that three cases not fit them.

The irregularities observed in mitosis were regarded as the cause for the DI increase in the specimens cytological diagnosed as tumours (Hedley et al., 1987; Keren, 1994; Harvey et al., 1993; Merkel and McGuire, 1990; Shapiro, 1989; Dowle et al., 1987; Daidone et al., 1995). The results from the present study with the FCM applications are consistent with previous studies. In the advanced stages of breast cancer and in metastasis, changing from the diploidy to polyploidy, (Merkel and McGuire, 1990; Shapiro, 1989; Dowle et al., 1987; Auer et al. 1980; Dressler et al., 1992). In the another study, diploid in small tumours and polyploidy in larger ones have been reported (Dowle et al., 1987). Additionally, it has been reported that the use of the DI values in tumour classification is (regarding the breast tumours) more reliable than the use of the S phase values (Throud et al., 1986).

Some researchers hold that DI values in tumour diagnosis can be referred to as a criterion and add that FCM appears more advantageous than microcytopho-

tometry (Harvey et al., 1993). Considering the research up to date and the diversity of results of the studies using the FCM, it is possible to regard our study with the three exceptional cases as in harmony with the previous research. In this respect, the DI values attained through the FCM measurements are apparently conductive to the histopathological tumour diagnosis. Yet, it is equally apparent that FCM alone does not suffice for the diagnosis.

In this study, it appears that the means of G_1+G_0 of tumours group are significantly different (decreasing) than the normal group (Figure 3). The G_0 cells turn into G_1 cells of the growing tumours G_1+G_0 cells. Another cause for this change in G_1+G_0 cell population is, the period of transition from the G_1 phase to the S phase is shortened, which means the speed of transition from the G_1 phase to the S phase is increased. In terms of cell physiology, it can be inferred that the cells at the G_1 phase go through a rapid transition to the S phase. And thus, this cause tumour occurrence. At the same time, an increase can be thought to take place in the transition from the S phase to the G_2+M phase. This (and in relation to the increase in number of cells at S phase) brings about the condition whereby the metabolic needs of the cells cannot be met. (Eskelinen et al., 1989b; Longin et al., 1992; Ferno et al., 1992). As another cause, the speed of the mitosis activity decreases in tumours cell populations after a while, transition from the M phase to the G_1 phase decreases end it can be thereby expected to be observed a corresponding decrease with the quantity of G_1+G_0 cells.

In this research, it was observed that the mean values of S phase of tumour cells increased (Figure 3). The cause of this result was, the most of the G_0 cells among the G_1+G_0 cells in the tumour cell population turned into G_1. Another cause is considered to be the increase in the S phase due to the decrease in the speed of transition from the S phase to the G_2 phase.

The reason of the increase in the G_2+M ratio is that tumour cell population is formed highly by the cells in the S phase which, brings about the condition that the quantity of the cells that enter the G_2+M phase increased (Figure 3). In addition, some of the cells which came up to the level of mitosis in the tumour cell population remain in cytokinesis, which causes that the cells in the G_2+M phase increase in number.

The findings in this study revealed that cytological method as well as FCM can be applied to determine the cell populations which may form normal and abnormal tissues. DI-show that FCM can be reliably employed as a method in early diagnosis of the tumours, in monitoring the growing illness and in the direction of the treatment. Still, in order to provide increased reliability and standardisation, the base and peak limitations of the FCM should be clarified and well defined. Since, blurring limits the human errata based on subjectivity, the much expected assure a definite reliability, a common evaluative system should be established and put to use. For achieving more reliability with the FCM method, determining the change in the ratio of the G_0 cell among the G_1+G_0 cells within the cell populations forming the normal and abnormal tissues (by referring to in-vitro or in-vivo studies) can be considered to be a progressive step. A treatise in point would much contribute to our cause by eliminating the hitherto attested shortcomings in the area.

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