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MicroRNA95 May Be Involved in Oligometastatic Prostate Cancer

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Abstract: The oligometastatic status in the prostate is a new entity of metastatic patients in which their treatment allows to improve survival over standard treatments. There are several theories about their biological origin, one of them being alterations in the expression of miRNas This. was a retrospective multicentre study undertaken in patients with oligometastatic prostate cancer who were diagnosed and treated at one of 7 different Spanish healthcare centres. METHODS: The study included 22 patients; healthy and primary tumour biopsy tissue was analysed in 7+2 of them in order to determine if they had a characteristic microRNA expression profile. We quantified the expression of the following miRNAs: mir-200a, mir-200b, mir-200c, mir-210, mir-95, mir-96, mir-654-3p, mir-543-3p, mir-21, mir-16-5p, mir-191-5p, and mir-93-5p, with the latter three being endogenous-expression controls. RESULTS: Our results show that miRNA95, and to a lesser extent, miRNA654-3p, were significantly underexpressed (or their expression was suppressed) in tumour tissue samples compared to normal perilesional tissue in all our patients; miRNA95 was underexpressed in 67% of the patients in our sample However, we detected no relationship between miRNA95 expression and the Gleason scores obtained for our patients. CONCLUSIONS: The simple size in our series are limited, but they do allow us to infer that there could be a specific miRNA expression signature in oligometastatic patients with prostate cancer, which may be of great interest in the development of future clinical trials and subsequent studies.

Keywords: Microrna, Paraffin Blocks, Prostate Cancer, MetÁstasis Biology, Oligometastasis

1. Introduction

Prostate cancer is the most common cancer among men. In the United States, with an expected 180,890 new cases per year, prostate cancer is the second leading cause of death from cancer in men. For men diagnosed with metastatic prostate cancer, the 5-year survival rate is as low as 28% [1]. Unlike other diseases, in prostate cancer there is still no clear scientific evidence that treating oligometastases impacts survival, although recent articles such as those by Ost et al. [2] have established that among patients treated with hormonal therapy, disease-free survival is higher in individuals whose therapies are aimed at oligometastases. Biochemical responses were obtained in these patients, which delayed disease progression and therefore, also delayed the use of systemic therapies, Kim [3] Philips [4]. Very recently, the results of the phase-II SABR-COMET [5] trial have shown that patients whose oligometastases were treated had a 5-year survival rate almost twice the length of patients in the control group.

Several clinical trials are currently underway [5, 6-11] which aim to fundamentally advance this area of research in terms of quality of life and survival. Current clinical guidelines like those from the National Comprehensive Cancer Network already collect different classification data from within stage M1 (metastatic) diagnoses, based on whether these metastases are found in lymph node, bone, or visceral locations. Indeed, clinical criteria are available (which are primarily used in current trials) such as those used by Tree et al [12], Kneebone or Bowden [13, 14] that consider oligometastatic patients with up to 3 metastases. This contrasts with other pathologies where clear treatment choices have already been established for oligometastatic situations, such as tumours originating at the lung [15] or colorectal level [16].

There are several theories about the biology of metastasis, starting from the work by Paget et al [17] with his 'seed and soil hypothesis', to the proposals by Hellman and Wiesselbaum [18] and Nguyen et al [19]. Other hypothesises have subsequently appeared, such as those by Gangaraju et al or Leong [20, 21] which involve a role for microRNAs (miRNAs). MicroRNAs are small chains of non-coding endogenously-expressed RNAs (20–25 nucleotides long) that regulate post-transcriptional gene expression. More than 1,500 miRNAs have been identified so far in humans. They have regulatory functions in many biological processes such as cell proliferation, differentiation, survival, apoptosis, and stress responses.

MicroRNAs are also involved in the onset and progression of cancer in humans where they function as potential oncogenes and tumour gene suppressors. They have been shown to modulate protein expression levels by recognition of target mRNAs by hybridising with them and inhibiting their translation. Interestingly, the mRNA translation of a gene-coding sequences can be regulated by several miRNAs and moreover, miRNAs can control the translation of multiple mRNAs from different genes. Authors including Baranwal, Li or Liu, et al. [22-24] posit that these attributes mean that miRNAs play a fundamental role in cell migration, invasion, and proliferation and so, they could act as cancer promoters or suppressors.

Studies such as that by Schaefer, Xu or Nabayi, et al [25-27] have established the origin of these 'aberrant' levels of miRNAs in tumour cells by comparing healthy prostrate tissues to those from prostate tumours. MicroRNA expression control is dysregulated in tumours, which results in miRNA upregulation or downregulation. In addition, in the case of prostate cancer, this dysregulation has also been associated with classic clinicopathological parameters such as prostate-specific antigen (PSA) and Gleason scores. Thus, the aim of this retrospective study was to analyse healthy and primary prostate tumour biopsy tissue from patients with oligometastatic prostate cancer diagnosed and treated in one of 7 Spanish healthcare centres in order to determine whether a characteristic miRNA expression profile exists in this patient subgroup.

2. Methods

We analysed samples from 9 patients with a mean age of 68.2 years (range: 60–72 years; median 70.5 years) who had locally-advanced neoplasms (5 pT3A and 4 pT3B stages), Gleason scores between 6 and 9 (one Gleason 6, four Gleason 7, and four Gleason 9), and mean PSA figures of 11.6 ng/dl (range: 6.6–22 ng/dl; median 8.7 ng/dl). A total of 22 patients with oligometastatic prostate carcinoma were included in the study. The Molecular Biopathology Services at each patient's diagnostic hospital received the samples from each of the 9 patients who had been treated with radical prostatectomy as paraffin blocks, as well as from 13 samples obtained from transrectal needle biopsies.

The blocks were cut with a microtome, and four 5-micron-thick cuts were fixed on a slide; one of these cuts was stained with haematoxylin-eosin and was evaluated by a pathologist in order to identify a tissue area rich in tumour cells. The other three cuts were re-paraffinised by covering the entire lamella, including the entire surface of the tissue section, with a thin layer of paraffin (used to avoid oxidation). The blocks were between 2 and 9 years old. The tumour area was defined within the paraffin sections as areas where at least 80% of the cells were cancerous and the proportion of tumour and perilesional tissue ranged between 80%–20%; sections where the cells were 100% cancerous (two cases) were not considered. Moreover, the diameter of transrectal needle biopsies contained in paraffin sections (less than 1 mm) and the proximity of the multiple samples from the same patient needle biopsy contained within these sections made it very difficult to separate tumour tissue from normal tissue. Therefore, we decided to exclude these 13 cases from the study.

We extracted RNA from the paraffinised tissues using a RecoverAll™ Total Nucleic Acid Isolation kit (Invitrogen,

Thermofisher Scientific). To do this, we separately isolated the RNA from tumour tissue and perilesional tissue microdissected from 7 of the radical prostatectomy samples; for the remaining two samples, we were only able to isolate RNA from tumour tissue because the cut area lacked non-tumour tissue. The concentration and purity of the RNA we obtained was evaluated using a BioSpec-nano spectrophotometer (Shimadzu). The RNA concentrations ranged from 1.6 ng/µl to 46.7 ng/µl (mean: 10.3 ng/µl) and the purities measured by the A 260/280 nm absorbance ratio were between 1.7 and 2.7 (mean: 2.1). For the relative quantification of the expression of our miRNA panel, we carried out RNA to cDNA reverse transcription with subsequent pre-amplification, using the TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystems) using a GeneAmp™ PCR System 9700 thermocycler (Applied Biosystems). We then used these cDNA samples to perform relative quantitation real-time QPCR.

Pre-amplification PCR reactions were started using 3.4 to 23.8 ng of RNA. These pre-amplified cDNAs were then diluted in ultrapure water (at a ratio of 1:5 to 1:30) and used for real-time QPCR, starting with 7 ng of RNA (range: 5.6 ng to 8.6 ng) using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and SDS (version 2.4) software. For miRNA expression quantification we used a TaqMan Fast Advanced Master Mix kit with predesigned primers and probes corresponding to the TaqMan Advanced miRNAs Assays (Applied Biosystems) for the following miRNAs: mir-200a, mir-200b, mir-200c, mir-210, mir-95, mir-96, mir-654-3p, mir-543-3p, mir-21, mir-16-5p, mir-191-5p, and mir-93-5p. The thermocycler parameters for the QPCR were: enzyme activation and DNA denaturation (95°C for 20 s), followed by 40 cycles of denaturation (95°C for 3 s), and hybridisation and extension (60°C for 30 s). The total volume of each PCR reaction was 20 µl.

Given the robustness of our results, the QPCRs were performed in duplicate for each sample/miRNA and the means of the miRNA expression quantification results were compared by normalising their expression levels to an endogenous mir-16-5p control miRNA. This miRNA was chosen because its levels of expression were the most consistent of the three control miRNAs used in the study. The expression levels were analysed using the $2^{-\Delta\Delta Ct}$ relative quantification method, using RQ Manager (version 1.2) software.

The miRNAs analysed were: miRNA200a, 200b, and 200c, miRNA210, miRNA95, miRNA96, miRNA654-3p, 542-3p, and miRNA21; and the endogenous-expression controls were miRNA 16-5p, miRNA 191-5p, and miRNA 93-5p. We analysed a total of 16 samples (9 from tumours and 7 normal control samples). The $2^{-\Delta\Delta Ct}$ expression values were used for paired normal tissue versus tumoural tissue data analysis for the 7 patient cases where both tumour and normal tissue were available. For the two cases for which only the tumour tissue samples were available, the normal control tissue was replaced with healthy tissue from another patient whose sample was analysed on the same PCR plate.

Statistical analysis was carried out with IBM-SPSS (version 22) software based on the results obtained with the RQ Manager (version 1.2) software. The descriptive values are expressed as the mean plus their standard deviation and the median and their minimum and maximum values.

3. Results

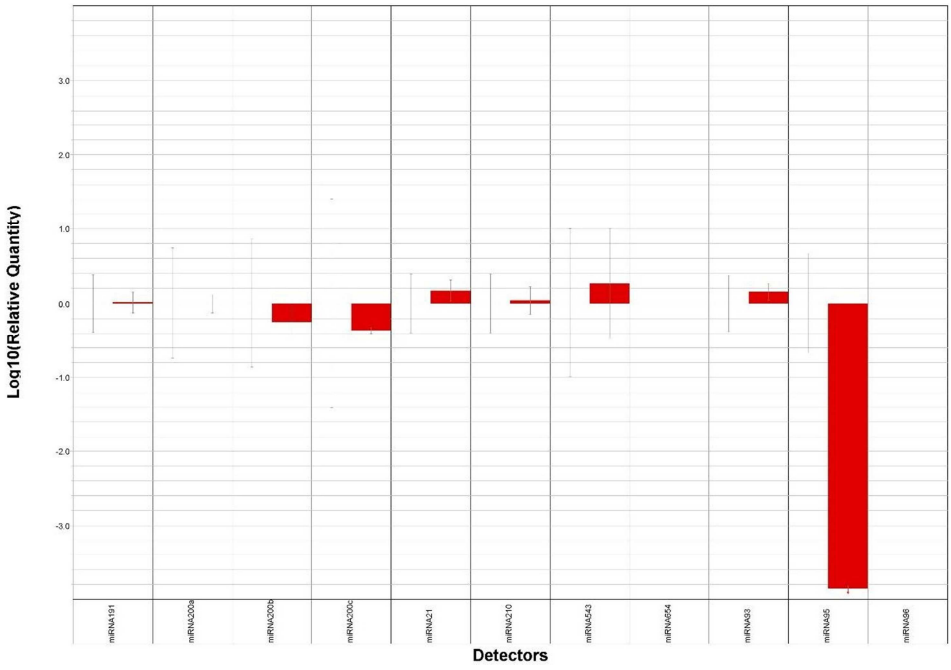

Figure 1. *The relative expression between normal and tumour tissue from study biopsy number 2 (2T and 2N biopsies), using miRNA16-5p as an endogenous control for the normalised expression of all the miRNAs analysed in this study. Of note, miRNA95 was underexpressed by more than 10^{-4}-fold. The data were obtained using RQ Manager (version 1.2) software from a 7900HT Fast Real-Time PCR System (Applied Biosystems) based on the $2^{-\Delta\Delta Ct}$ method.*

Our results show that miRNA95, and to a lesser extent, miRNA654-3p, were significantly underexpressed (or their expression was suppressed) in tumour tissue samples compared to normal perilesional tissue in all our patients. Of the 9 patients we studied, miRNA95 expression in the tumour samples was more than 10−4-fold less than in the controls in 4 (44.4%) cases (Figure 1) and, in 2 (22.2%) patients, its expression was up to 8 times lower than in the control samples (Figure 2). MicroRNA95 was not expressed in tumour or healthy tissue in 2 patients (22.2%), and in 1 case (11.1%), its expression in tumour tissues was approximately double that of the control. In other words, miRNA95 was underexpressed in 67% of the patients in our sample (Figure 3). However, we detected no relationship between miRNA95 expression and the Gleason scores obtained for our patients. As shown in Table 1, miRNA95 was not expressed in healthy or tumour tissue samples in 2 patient biopsies (cases 5T-5N and 6T-6N); additionally, miRNA 654-3p was not expressed in healthy or tumour tissues in 6 patient biopsies.

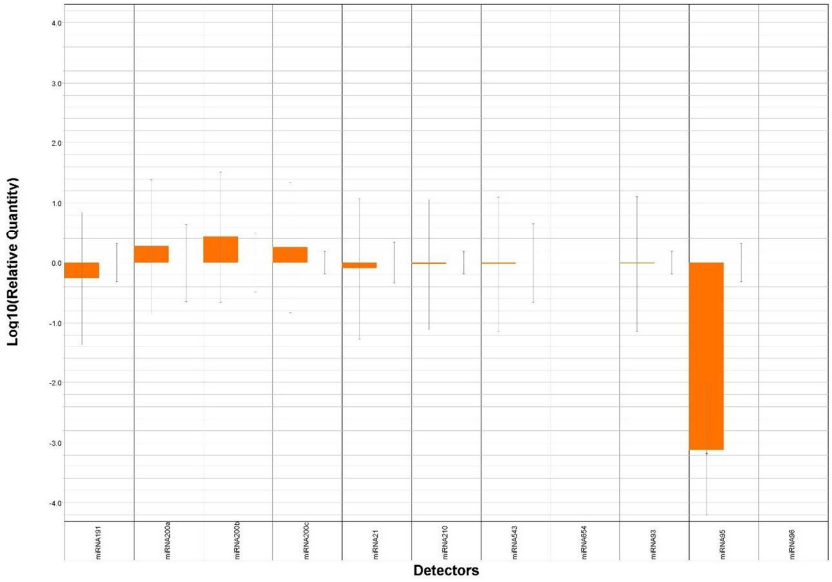

Figure 2. *The relative expression between normal tissue from one patient and tumour tissue from another patient in the study (4T and 14N biopsies) using miRNA16-5p as an endogenous control for the normalised expression of all the miRNAs analysed in the study. Of note, miRNA95 was underexpressed by more than 10^{-3}-fold. The data were obtained using RQ Manager (version 1.2) software from a 7900HT Fast Real-Time PCR System (Applied Biosystems) based on the $2^{-\Delta\Delta Ct}$ method.*

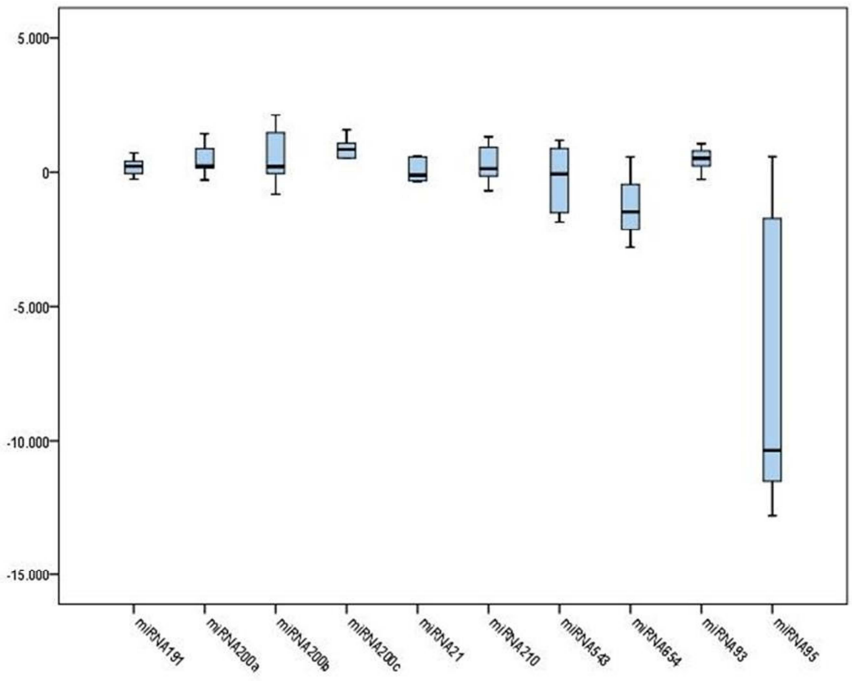

Figure 3*. Mirna95 was significantly underexpressed in prostatic oligometastatic tumours (by more than 10^{-4}-fold). The data shown correspond to those presented in Table 1.*

Table 1. *The relative variation in expression (using the $2^{-\Delta\Delta Ct}$ method) between normal and tumour tissues for all the miRNAs analysed in the study, normalised to miRNA16 expression. MicroRNA95 was significantly underexpressed, by a median of more than 10−4-fold in oligometastatic tumour tissues with respect to normal tissue.*

	N						
	Valid	**Missed**	**Mean**	**Median**	**Desv. Std.**	**Min.**	**Max.**
EXPRESSIONmiRNA191	9	0	121,56	220	475,29	-849	712
EXPRESSIONmiRNA200a	9	0	462,78	235	542,16	-290	1422
EXPRESSIONmiRNA200b	9	0	597,33	212	1054,08	-816	2135
EXPRESSIONmiRNA200c	9	0	574,11	845	840,70	-1199	1574
EXPRESSIONmiRNA21	9	0	58,67	-109	420,90	-351	601
EXPRESSIONmiRNA210	9	0	89,67	133	979,92	-1870	1309
EXPRESSIONmiRNA543	9	0	-250,44	-67	1230,53	-1842	1180
EXPRESSIONmiRNA654	3	6	-1234,33	-1465	1699,78	-2807	569
EXPRESSIONmiRNA93	9	0	486,67	518	454,39	-264	1051
EXPRESSIONmiRNA95	7	2	-7007,57	-10353	5770,20	-12800	574

4. Discussion

In the McDonald or Sita-Lumsden et al [28, 29] studies, there were lower expressions of miR-28, miR-100, miR-942 and miR-28-3p, and higher expressions of miR-708, miR-1298, miR-886-3p, miR-374, miR-376c, miR-202, miR-128a, miR-185 and miR-21; expression levels increased significantly in both plasma samples from patients with more advanced and localized prostate cancer. Sita-Lumsden specifically evaluated miR 21 in patients with localized prostate cancer, androgen-dependent prostate cancer (ADPC), castration-resistant prostate cancer (CRPC) and benign prostatic hyperplasia, and found that miRNA21 levels were significantly higher in patients with CRPC or ADPC and PSA levels higher than 4 ng/ml. However, in our study we did not detect any change in the expression of this miRNA.

Walter et al [30] studied the role of determinated miRNAs IN 37 patients, as biomarkers in low- intermediate and high grade prostate tumors, by studying them in two groups: those with Gleason scores of 6 and 7 (3+3 and 3+4) and those with high-grade tumours with Gleason scores of 8 and 9 (4+4 and 4+5), compared to normal stromal cell epithelium. These authors found different expression patterns in high-grade tumours (Gleason = 8) compared to tumours with a Gleason score of 6, and observed overexpression of miR-122, miR-335, miR-184, miR- 193, miR-34, miR-138, miR-373, miR-9, miR-198, miR-144, and miR-215, and underexpression of miR-96, miR-222, miR-148, miR-92, miR-27, miR-125, miR-126, and miR-27 in high-grade tumours. In our series of oligometastatic patients, we detected no differences in the expression of these miRNAs between the different patient Gleason-index scores.

Nam et al [31] studied the profile of miRNa in 28 patients who developed metastases after a prostatectomy, detecting an increase in miRNa-200a regulation, WHICH DATA IS NOT COINCIDENT IN OUR series.

Li et al [32] in a study with more than 300 patients presented results demonstrating that miRNA95 was downregulated in simple tumors but did not study its relationship with metastasis, correlating with the pathological postoperative state T/N. They considered that miRNA-23b, miRNA-95 miRNA-143 and miRNA-183 can be used to help the diagnosis and prognosis of prostate cancer as biomarkers. Our series shows that miRNA95 was underexpressed in oligometastatic patients coinciding with the results of Li. However, no relationship was detected between miRNA95 expression and Gleason scores, although this may be related to the small size of our study sample. McDonald (28) in a peripheral blood study detected correlation with a high Gleason index and underexpression of some miRNA, not finding miRNA95 among them. Stuopelyte et al [33] correlated the overexpression of miRNA95 in prostate cancer cells with the presence of locally advanced tumors (stage T 3) and tumors poorly differentiated according to Gleason scores. We could not corroborate this finding in our series, however, as far as we know, ours is the first study to find evidence of miRNA95 underexpression in patients with oligometastatic prostate cancer

Other authors including Ma et al [34] observed increased miRNA95 expression in relapsed cases. In gliomas, Fan [35] correlated the miRNA95 underexpression with less proliferative and invasive capacity in glial tumors, consolidating its use as a prognostic marker and thus individualizing the treatment according to the risk of progression.

The miRNA-200 family and s plays a central role in epithelial cell plasticity and performs functions controlling cell invasiveness, stemness and tumour metástasis -Gibons et al [36]). Lussier [37] observed increased miRNA200c expression in metastasic patients, analysing tissue samples from 42 patients with oligo- or polymetastatic cancer. These authors observed that miRNA200-c expression was more common in tumour tissue from oligometastatic patients who had progressed to a polymetastatic stage, therefore suggesting that miRNA200-c may play a role in controlling the passage of cancer from an oligo- to polymetastatic status. MicroRNA654-3p behaved similarly.In miRNA542 3p an increase was observed only in primary tumor tissue and not in metastatic tissue

In the analyzed series we did not observe any alteration in the expression of these families (miRNA 200, miRNA542 3p) and in some cases, mRNA654-3p was underexpressed.

Other authors [38, 39] find that overexpression of miRNA96 was an independent prognostic factor for recurrence. In addition, no overexpression of miRNA96 was detected in our series, although oligometastases occurred as part of the evolution of these patients. This indicates that miRNA96

expression could be a prognostic indicator of biochemical relapse or polymetastasic disease, but not of oligometastasis

Formosa et al [40], have correlated the overexpression of miRNA654 3p with a suppressor effect in inhibiting cell migration and the invasiveness of prostate cancer cell lines. Lu et al [41] found an overexpression of miRNA-654 in oral squamous cell carcinoma correlating with aggressiveness, invasion and metastasis. Geraldo et al [42] detected in a murine model of papillary thyroid carcinoma that restoration of miR-654-3p expression using commercial mimetic miRNA significantly reduced proliferation and celular migration, and increased apoptosis in normal and tumor. In our study, miRNA654 3p is infraexpressed to a less measure than miRNA95.

The miRNA654 3p overexpression, related to androgenic receptor regulation [43], is associated with cell proliferation and migration in several articles [40, 41, 42], but has not been ratified in oligometastatic prostate cancer, so future studies are required to analyze its role.

5. Conclusions

Despite the limited data available, in our series it is likely to infer that there is a signature expression of miRNA in patients with oligometastatic prostate cancer, an aspect of great interest for future studies. However, advances in molecular biology techniques and the possibility of making determinations in urine [44]- or simple plasma – [45]- open new horizons for the future study of highly specific groups of patients with oligometastatic prostate cáncer.

In conclusion, miRNA95 underexpression in tumor cells versus overexpression in healthy tissue suggests a suppressive function in oligometastasic prostate cancer, but larger sample size studies are needed to confirm our results.

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Compliance with Ethical Standards

This project was approved by the Clinical Research and Ethics Committee at the Provincial Hospital Consortium of Castellón (Spain) in 2015; Informed consent from all the study participants was obtained in accordance with Article 58.2 of Spanish Biomedical Research Law (Law 14/2007).

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