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This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other peer-reviewed media. All authors listed have contributed sufficiently to the work to be included as authors. No conflict of interest, financial or other, exists.

Sincerely,

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Abstract

Alpha-Smooth muscle actin (α-SMA) is used as a marker for a subset of activated fibrogenic cells, myofibroblasts, which are regarded as important effector cells of tissue fibrogenesis. We addressed whether ASMA gene (actin alpha 2 gene or ACTA2) expression is upregulated in in cases with BM fibrosis compared to those with no BM fibrosis in order to evaluate its role in the pathogenesis of BM fibrosis. ASMA expression was detected by quantitative RT-PCR in formalin fixed paraffin embedded (FFPE) bone marrow trephine biopsy samples of cases with neoplastic bone marrow diseases as well as reactive bone marrow disorders cases. Both groups included cases with and without bone marrow fibrosis. Results indicated that there was no statistically significant difference in ASMA (ACTA2) gene expression between neoplastic fibrotic and non-fibrotic cases as well as between reactive fibrotic and non-fibrotic cases. Also the level of α-SMA expression does not correlate positively with the grade of bone marrow fibrosis. We conclude that α-SMA alone can’t be considered a functional marker of fibrogenic cells in bone marrow fibrosis. Exploration of other related genetic pathway is recommended.
• Introduction

Bone marrow (BM) fibrosis is the continuous replacement of blood-forming cells in the BM by excessive scar tissue, leading to failure of the body to produce blood cells and ultimately to death (Gleitz et al., 2018).

A number of haematological and non-haematological disorders are associated with increased BM fibrosis, including myelodysplastic syndromes and myeloproliferative neoplasms (MPNs) (Kuter et al., 2007).

Fibrosis is a wound healing response to injury and characterized by excessive synthesis and accumulation of extracellular matrix (ECM) proteins in many organs such as skin, liver, lung, kidney, and heart, which ultimately leads to organ failure and death (Rockey et al., 2015).

Pathologically, myofibroblasts are the major cellular source of ECM in most forms of fibrogenesis, despite the fact that myofibroblasts may originate from various cell types depending on the organ and mechanism of injury (Hinz et al., 2007).

Nevertheless, myofibroblasts share two common features de novo expression of smooth muscle a-actin (SM a-actin) and abundant ECM, particularly type 1 collagen (COL1A1) (Rockey et al, 2019).

Actins consist of six isoforms and play an essential role in many cell processes, including cell proliferation, differentiation, migration, and contraction (Sandbo et al., 2011).

**Ueyama et al. (1984)** isolated and characterized the Actin Alpha 2 (ACTA2) gene, encoding smooth muscle aortic actin. They found that the ACTA2 gene contains at least 9 exons.
Alpha smooth muscle actin appears to play an important functional role in myofibroblast contraction and migration. Previous studies have shown that stress fibres containing SM a-actin, not only generate greater contractile force than stress fibres that contain only cytoplasmic-b and g-actin, but also associate with supermature focal adhesions, which in turn exhibit a specific molecular composition of integrins avb3 and a5b1 (*Goffin et al., 2006*) (*Rockey et al., 2013*).

Integrins are transmembrane proteins, and thus integrins can bidirectionally transmit the mechanical signal across the cell membrane (*Harburger et al., 2009*).

Although the molecular mechanisms by which the mechanical signal, transmitted by Integrins associated with α-SMA, regulates extracellular matrix expression remain unclear, studies have revealed that the cell contractile force (mediated by actin stress fibres), as a mechanical signal, could be transformed to a chemical signal to modulate ECM genes such as COL1A1 expression through transforming growth factor-b (TGF-b) and Erk signal pathways (*Shi et al., 2017*).

Although multiple evidence supports that ASMA plays an important role in multiple features of myofibroblasts, its role in fibrogenesis remains controversial (*Takeji et al., 2006*).

To shed light on this question, our study evaluated the degree of ACTA2 gene expression in cases with BM fibrosis compared to those with no BM fibrosis in order to evaluate its role in the pathogenesis of BM fibrosis and thus defining patients that could potentially benefit from ASMA-targeted therapy.
• **Study design and protocol**

This study included collection of archived formalin fixed-paraffin embedded bone marrow trephine biopsies of patients with different underlying diseases from the bone marrow pathology unit, Clinical pathology department, Faculty of medicine, Cairo University during the period from June 2016 to October 2017.

RNAs of 90 adequate samples were used to complete the study. Those included 45 cases with neoplastic bone marrow diseases and 45 cases with reactive bone marrow disorders.

• **Key inclusion criteria**

Any archived bone marrow core biopsy that fulfills the fixation and processing requirements for the molecular study.

• **Key exclusion criteria**

We excluded bad archived trephine bone marrow biopsy samples and those which are not adequately processed.

• **Methodology**

Cases of different underlying pathology are subjected to the following laboratory techniques:

1- **Provisional selection of the samples** with full data recording

2- Bone marrow samples were stained for Reticulin (Silver impregnation) according to *(Bain et al., 2010)* & trichrome according to *(Peterson and Brunnin, 2001)*. Evaluation of fibrosis grade is assessed according to the 2016 WHO grading of BM fibrosis.(ref.)

According to the underlying bone marrow conditions cases were divided into 2 groups:
**Group 1:** Neoplastic group that included 45 cases with neoplastic bone marrow disorders (acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPNs), lymphoproliferative and plasma cell disorders).

**Group 2:** Reactive group that included 45 cases with normal and reactive bone marrow changes (included cases of erythroid hyperplasia, hypercellular marrow with eosinophilia and increased megakaryocytes and sarcoidosis) and normal bone marrow as well as cases with miscellaneous conditions.

According to the bone marrow fibrotic status both groups were further divided into 2 subgroups using 2008 WHO grading of BM fibrosis (revise as above).

Fibrotic subgroup included cases with bone marrow reticulin fibrosis grade > 2.

Non fibrotic subgroup included cases with ≤ grade 2 reticulin fibrosis.

3- Real-time PCR.

Total RNA was extracted from bone marrow trephine biopsy samples using ready-made commercial kit (RNeasy FFPE Kit, cat no. 73504) according to the manufacturer's protocol. Isolated RNA samples were then used for RT-PCR. Samples of total RNA (1 μg) were reverse transcribed into cDNA in 20 μl reaction volumes using a first-strand cDNA synthesis kit for RT-PCR according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA). Gene expression levels were quantified by real-time PCR using a SensiMix SYBR kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The primer sequences used for this analysis were as follows: human α-SMA (forward, 5'-AGA CAT CAG GGG GTG ATG GT-3' and reverse, 5'-CAT GGC TGG GAC ATT GAA AG-3'), and GAPDH
was used as an internal control (forward, 5'‑ATT GTT GCC ATC AAT GAC CC‑3' and reverse, 5'‑AGT AGA GGC AGG GAT GAT GT‑3'). An annealing temperature of 60°C was used for all the primers. PCR was performed in a standard 96‑well plate format with an ABI 7500HT Real‑Time PCR Detection System (Thermo Fisher Scientific, Inc.). For data analysis, the raw threshold cycle (CT) value was first normalized to the housekeeping gene for each sample to obtain a ΔCT. The normalized ΔCT was then calibrated to the control samples and to obtain the ΔΔCT values.

**Statistical analysis**

Data management and statistical analysis were done using SPSS v.25. Numerical data were summarized using mean and standard deviation or median and range. Categorical data was summarized using numbers and percentage. Different levels of gene expression were compared using Mann‑Whitney test. Correlation analysis was done between different levels of gene expression and degree of reticulin bone marrow fibrosis using spearman correlation. “r” is the correlation coefficient. It ranges from -1 to +1. -1 indicates strong negative correlation. +1 indicates strong positive correlation. All P values were two sided. P values less than 0.05 were considered significant.

- **Results**

**Baseline characteristics of the study population:**

According to the underlying bone marrow conditions cases were divided into 2 groups:
**Group 1:** Neoplastic group that included 45 cases with neoplastic bone marrow disorders

**Group 2:** Reactive group that included 45 cases with normal and reactive bone marrow changes

According to the bone marrow fibrotic status both groups were divided into 2 subgroups:

The Neoplastic group included 26 (57.8%) trephine bone marrow samples belonging to fibrotic subgroup and 19 (42.2%) belonging to non-fibrotic subgroup. While the reactive group included 17 (37.7%) samples belonging to fibrotic subgroup and 28 (62.3%) samples belonging to non-fibrotic subgroup.

**Gene expression findings**

On comparing fibrotic versus non-fibrotic subgroups in the neoplastic as well as in the reactive groups median ACTA2 gene expression showed no statistically significant difference (P value = 0.291, 0.314 respectively) **Table 2.**

No statistically significant correlations were detected between ACTA2 gene expression and the grade of fibrosis in all studied cases.

- **Discussion**

Fibrosis and resultant organ failure account for at least one third of deaths worldwide. Since fibrosis is common and has adverse effects in all organs, it is an attractive therapeutic target (*Zeisberg and Kalluri, 2013*).

Although we understand many of the cellular and molecular processes underlying fibrosis, there are few effective therapies and fewer that target fibrogenesis specifically. These facts highlight the need for a deeper
comprehension of the pathogenesis of fibrogenesis and the translation of this knowledge to novel treatments (Rockey et al., 2015).

The present study showed no statistically significant difference in ACTA2 gene expression levels in cases with bone marrow fibrosis compared to those with no bone marrow fibrosis neither in the neoplastic nor in the reactive groups.

In concordance with these results Zhao et al., (2018) addressed whether α-SMA-expressing myofibroblasts are detectable in fibrotic muscles of mdx5cv mice, a mouse model for Duchenne muscular dystrophy (DMD), and whether the α-SMA expression correlates with the fibrogenic function of intramuscular fibrogenic cells. They found that α-SMA immunostaining signal was not detected in collagen I (GFP)-expressing cells in fibrotic muscles of Coll-GFP/mdx5cv mice, but it was readily detected in smooth muscle cells lining intramuscular blood vessel walls.

They concluded that myofibroblasts are present in fibrotic skeletal muscles, but their expression of α-SMA is not detectable by immunostaining. They also reported that the level of α-SMA expression by intramuscular fibrogenic cells does not correlate positively with the level of collagen gene expression or the severity of skeletal muscle fibrosis in the mdx5cv mice and they concluded that α-SMA is not a functional marker of fibrogenic cells in skeletal muscle fibrosis associated with muscular dystrophy (Zhao et al., 2018).

In contrast to our findings, Varma et al., (2016) selected 32 pairs of baseline and follow-up liver biopsies taken from stable pediatric liver transplant recipients and performed morphometric quantification of “ASMA positive area percentage” on the baseline biopsy as well as histological and fibrosis assessment using Metavir and liver allograft fibrosis scores (LAFSc) on all biopsies.
They reported that significant association was seen between extent of ASMA positivity on baseline biopsy and “prospective change in fibrosis” using Metavir (p value = 0.02), cumulative LAFSc (p value = 0.02), and portal LAFSc (p value = 0.01) values. ASMA positive area percentage >1.05 predicted increased fibrosis on next biopsy with 90.0% specificity. They came to the conclusion that ASMA quantification can predict the future course of fibrosis after liver transplantation.

Another study by Rockey et al., (2019) investigated the relationship between ASMA and type 1 collagen expression (COL1A1), a major extracellular matrix protein important in liver fibrosis with the results demonstrating that knockout of ACTA2 gene leads to reduced liver fibrosis and COL1 expression.

These differences can be justified in part by differences in the technique used to detect ASMA expression and the difference in the tissue used. Also in vitro culture can alter the gene expression. In the future, it will be important to carry out more genetic lineage tracing experiments of stromal cell populations to identify the source of active myofibroblasts in fibrosis and precisely characterize changes in cellular behavior over time.

**Conclusion**

In summary, we have extensively investigated whether α-SMA plays a role in pathogenesis of bone marrow fibrosis and whether it can be used alone as a marker of pathologic fibroblasts affecting the bone marrow. Our results suggest that α-SMA alone, can’t be considered an independent markers in bone marrow fibrosis and doesn’t probably play a process-limiting role for both contractile and collagen-producing fibroblast. Exploration of related genetic pathway is recommended for this purpose.

**References**
1. Tables

Table 1. Number and percentages of cases in each subgroup.

<table>
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<tr>
<th></th>
<th>Neoplastic (total number)</th>
<th>Reactive (total number)</th>
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<tbody>
<tr>
<td></td>
<td>Fibrotic</td>
<td>Non-fibrotic</td>
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<tr>
<td>Number (%)</td>
<td>26 (57.8%)</td>
<td>19 (42.2%)</td>
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Table 2. Comparison of ACTA2 gene expression between fibrotic and non-fibrotic subgroups in both neoplastic and reactive groups.

<table>
<thead>
<tr>
<th></th>
<th>Neoplastic</th>
<th>P</th>
<th>Reactive</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td>Fibrotic</td>
<td>Non-fibrotic</td>
<td>Fibrotic</td>
<td>Non-fibrotic</td>
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<tr>
<td></td>
<td>(total number)</td>
<td>(total number)</td>
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<tr>
<td>ACTA2</td>
<td>0.04</td>
<td>0.03</td>
<td>0.314</td>
<td>0.02</td>
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<td>(0.01-0.42)</td>
<td>(0.02-0.39)</td>
<td>(0.01-0.40)</td>
<td>(0.00-0.38)</td>
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Table 3. Correlation between the degree of bone marrow fibrosis and ACTA2 gene expression in all studied cases.

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<td>ACTA2 gene</td>
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Figures

Figure 1. Number and percentage of the fibrotic and non-fibrotic subgroups in each of the neoplastic and reactive groups.

Figure 2. ACTA2 gene expression in fibrotic and non-fibrotic subgroups in each of the neoplastic and reactive groups.
Figure 1.

![Bar chart showing the percentage of patients in fibrotic and non-fibrotic subcategories for Neoplastic and Reactive conditions.]

- Fibrotic Neoplastic: 57.8%
- Non-fibrotic Neoplastic: 42.2%
- Fibrotic Reactive: 37.7%
- Non-fibrotic Reactive: 62.3%

Figure 2.

![Bar chart showing the median ACTA2 levels in fibrotic and non-fibrotic subcategories for Neoplastic and Reactive conditions.]

- Fibrotic Neoplastic: 0.04
- Non-fibrotic Neoplastic: 0.03
- Fibrotic Reactive: 0.02
- Non-fibrotic Reactive: 0.01