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Fibroblasts in Hypoxic Conditions Mimic Laryngotracheal Stenosis*

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Abstract

Objective—To elucidate the role of hypoxia and inflammatory pathways in the pathogenesis of iatrogenic laryngotracheal stenosis (iLTS).

Study Design—1) Examination of mucosal surface gene expression in human iLTS 2) *In vitro* comparison of normal and scar laryngotracheal fibroblasts under normoxic and hypoxic conditions

Setting—Tertiary care hospital in a research university, 2012–2016

Subjects and Methods—Brush biopsies were obtained from normal laryngotracheal tissue and scar in iLTS patients; gene expression was compared. Fibroblasts were isolated from normal and scarred trachea and grown *in vitro* in either a 1% O₂ or normoxic environment. Cell growth and gene and protein expression were compared. Statistical analysis utilized a multilevel mixed-effects model.

Results—Expression of IL-6 (Fold-change = 2.8, p<0.01), myofibroblast marker α SMA (Fold-change = 3.0, p=0.01), and MMP13 (Fold-change = 5.4, p=0.02) was significantly increased in scar biopsy samples compared to normal. Under hypoxic conditions *in vitro*, normal laryngotracheal fibroblasts proliferated significantly faster (n=8, p<0.01 each day). Expression of IL-6 (n=8, Fold-change = 2.6, p<0.01) increased significantly after 12 hours under hypoxia. Expression of α SMA (n=8, Fold-change= 2.0, p=0.03), COL1 (n=8, Fold-change = 1.1), and MMP13 (n=8, Fold-change = 1.6, p=0.01) increased significantly after 48 hours under hypoxia. Scar fibroblasts also proliferated significantly faster under hypoxic conditions, but did not display the same expression profile.

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Conflicts of interest: None

Conclusion—Human iLTS scar has a myofibroblast phenotype. Under hypoxic conditions *in vitro*, normal laryngotracheal fibroblasts can transdifferentiate into a similar phenotype. These changes may be mediated by IL-6, a fibrosis-related cytokine.

Keywords

Iatrogenic; Laryngotracheal stenosis; Hypoxia; Fibrosis; Scar; Subglottic Stenosis

INTRODUCTION

Laryngotracheal stenosis (LTS) is a fibrotic disease that leads to luminal narrowing of the larynx and/or trachea and may cause significant respiratory distress.¹ Iatrogenic LTS (iLTS) is the most common etiology and can affect more than 10% of critically ill patients after endotracheal intubation.^{1,2} The mechanisms leading to iLTS are unknown, but may involve an abnormal wound healing response similar to other fibrotic diseases.³ The initial intubation injury can cause inflammation, which leads to fibroblast differentiation, proliferation, and formation of a pathological scar.^{4–6}

Few investigations into the pathogenesis of iLTS have focused on fibroblasts and myofibroblasts, the end effector cells in many diseases of fibrosis.⁷ Myofibroblasts can be derived from fibroblasts, are rarely found in normal tissue, and possess a distinct pro-fibrotic phenotype that combines contractility with extracellular matrix (ECM) deposition.⁸ Recent studies have shown that compared to normal fibroblasts, fibroblasts isolated from laryngotracheal scar proliferate faster, exhibit metabolic changes, and express more collagen.⁹ This echoes the fibrotic phenotype observed in other organs. In cardiac fibrosis, fibroblasts transdifferentiate into myofibroblasts in response to ischemic injury, producing excessive ECM and a contracted scar.¹⁰ Similarly, hepatic cirrhosis and idiopathic pulmonary fibrosis (IPF) involve the transdifferentiation of hepatic stellate cells and pulmonary fibroblasts into myofibroblasts.^{11,12} In *in vitro* models from the heart, liver, lungs, and skin, a severe 1–3% O₂ hypoxic environment promotes the shift towards a myofibroblast phenotype, resulting in increased fibroblast proliferation and ECM production.^{13–16}

A few studies have explored the role of hypoxia in the pathogenesis of iLTS. Clinical experience shows that cuff pressure and endotracheal tube friction can both contribute to tracheal injury.¹⁷ This initial trauma can cause local tissue ischemia, especially in the critically ill and hypo-perfused patient.^{2,18} In prospective studies, severe respiratory failure and high cuff pressure correlated highly with tracheal injury.¹⁹ As the injury heals, local tissue may become even more hypoxic. Studies from hypertrophic skin scars show that oxygen tension in the skin during scar formation may be <10% of the baseline in normal skin.²⁰ In animal models of iLTS, piglets displayed histological signs of injury after 4 hours of intubation, and hypoxic ventilation significantly increased the severity of this injury.²¹

Yet despite the *in vitro* evidence from parallel fibrotic diseases in other organs, there has not been adequate investigation into the effect of hypoxia on laryngotracheal fibroblasts. Given the suspected mechanisms for local tracheal injury and ischemia, we hypothesize that hypoxia will promote a transdifferentiation of normal laryngotracheal fibroblasts into

myofibroblasts and help maintain the increased proliferation and ECM production observed in scar fibroblasts.⁹ We aim to first define the surface gene expression profile of laryngotracheal scar in one set of patients, and then test our hypothesis of myofibroblast transdifferentiation through an *in vitro* model, using fibroblast cell lines isolated from a separate set of iLTS patients.

METHODS

Subject recruitment

Informed written consent was obtained from all participants in accordance with the Johns Hopkins University Institutional Review Board (NA_00078310). All patients had a diagnosis of iLTS and received operative treatment from the senior author (ATH) between August 2012 and June 2016.

Extraction of RNA from human brush biopsy samples

Surface brush biopsies were obtained in the operating room from normal and scarred portions of the larynx and trachea of 9 iLTS patients using a ConMed Disposable Bronchial Cytology Brush (Utica, NY) visualized under direct laryngoscopy. All normal and scarred sites in the trachea were at least 2cm apart. Brush Samples were homogenized, and RNA was extracted using the AllPrep RNA/Protein Kit (Qiagen, Valencia, CA, USA). RNA quantification, cDNA synthesis, and quantitative Real Time Polymerase Chain Reaction (qRT-PCR) were conducted using technical triplicates.

Fibroblast isolation and culture

Tissue biopsies were subsequently taken from normal and scarred portions of the subglottis or trachea in 8 patients with iLTS (different than the brush biopsy group). Fibroblasts were cultured and expanded.²² After reaching P5-8, fibroblast cell lines were trypsinized, split, and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% GlutaMax (Gibco), 1% NEAA (Gibco), and 5% penicillin/streptomycin (Gibco) under either normoxic or hypoxic conditions. Normoxic cultures were incubated at 37°C in a humidified 5%CO₂/95% air incubator. Hypoxic cultures were incubated in a modular sealed, humidified, chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA) containing 1%O₂/5%CO₂/94%N₂ at 37°C; gas inside the chamber was replaced every 24 hours.

Cell proliferation and cell count

Fibroblasts in normoxic and hypoxic conditions were seeded onto 6-well culture plates (Falcon by BD Biosciences, Tewksbury, MA, USA) at 5×10³ cells per well. Cells were trypsinized and counted using a hemocytometer daily, over 6 days. All proliferation experiments were conducted in biological duplicates.

Gene expression analysis by quantitative Real-Time Polymerase Chain Reaction

Fibroblasts in normoxic and hypoxic conditions were seeded onto 6-well culture plates (Falcon) at 2.5×10⁵ cells per well. Cells were trypsinized after 12 and 48 hours. All *in vitro*

gene expression experiments were conducted in biological triplicates. RNA was extracted using RNAeasy Micro Kits (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). qRT-PCR was performed and monitored using the SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) on the StepOnePlus Real Time PCR System (Applied Biosystems). Reaction mixtures were incubated for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. ACTB was used as the reference gene. Target genes of interest included the fibrosis markers MMP13 (matrix metalloproteinase 13), COL1 (collagen 1), α SMA (alpha smooth muscle actin), FN1 (fibronectin 1), COL3 (collagen 3), as well as the inflammatory markers IL-6 (interleukin 6) and TGF- β (tumor growth factor beta). All primers are available upon request.⁹

Protein expression analysis by Western Blot

Fibroblasts in normoxic and hypoxic conditions were seeded onto T25 culture plates (Falcon) at 1×10^6 cells per plate. Cells were trypsinized after 12 and 48 hours. Protein was extracted using a lysis buffer consisting of RIPA buffer (50mM Tris-HCL, 150mM NaCl, 2% NP-40, 1% Na Deoxycholate, 0.2% SDS), 10 μ M PMSF, 50 μ M EGTA, 10 μ M Na Orthovanadate, 0.5 mM Na Fluoride, 1% cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) and deionized water. 20 μ g of proteins were resolved on NuPAGE 4–12% Bis-Tris gel (Life Technologies, Carlsbad, CA, USA) in NuPAGE MOPS SDS Running Buffer (Life Technologies) and transferred onto a PVDF membrane (Bio-Rad) using the NuPAGE Transfer Buffer (Life Technologies). The membrane was blocked for 1 hour using 5% bovine serum albumin (BSA, Sigma) in tris-buffered saline with 0.1% Tween (TBST), incubated at 4°C overnight with the primary antibody in TBST, washed, and finally incubated for 1 hour with the secondary antibody in TBST. Antibody concentrations were: 1° Mouse anti-GAPDH (abcam, Cambridge, MA), 1:3000; 1° Rabbit anti- α SMA (abcam), 1:200; 2° Goat anti-mouse (abcam), 1:5000; 2° Goat anti-Rabbit (Cell Signaling, Danvers, MA), 1:4000. Bands were visualized using the ECL Prime chemiluminescence system (ECL, Amersham, Freiburg, Germany) and detected using the UVP BioSpectrum Imaging System (UVP, Upland, CA, USA).

Statistical analysis

Cell proliferation results are expressed as the mean difference between groups $\pm$ 95% Confidence Interval (CI). The mean difference in cell growth on each day was compared between conditions (hypoxic vs. normoxic) using a multi-level mixed effects model in STATA12 (StataCorp, College Station, TX, USA).²³ Relative gene expression was calculated using the delta cycle threshold (dCT) method. In qRT-PCR, the CT value is defined as the number of cycles required for the fluorescent signal emitted by DNA amplification to cross a standard threshold. The dCT measures the amount of target DNA in the sample relative to the reference gene ACTB. dCT was thus defined as $CT_{\text{Target}} - CT_{\text{Reference}}$, and delta delta CT (ddCT) was defined as $dCT_{\text{Hypoxic}} - dCT_{\text{Normoxic}}$. Fold-change between hypoxic and normoxic conditions was calculated as 2^{-ddCT} .²⁴ dCT values between normoxic and hypoxic conditions were compared using a multi-level mixed effects model in STATA12. Statistically significance was defined as a p-value < 0.05 .

RESULTS

Human laryngotracheal scar displays increased expression of interleukin-6 (IL-6), α SMA, and MMP13 compared to normal tracheal mucosa

Demographic and clinical information for the surface brush biopsy iLTS patients (n=9) are displayed in Table 1. All surface brush biopsy iLTS patients were adults. Surface brush biopsies taken from scarred areas of the trachea showed significantly higher expression of IL-6 (Fold-change = 2.8, $p < 0.01$, 95% CI: 1.4–3.9), α SMA (Fold-change = 3.0, $p = 0.01$, 95% CI: 1.2–3.9), and MMP13 (Fold-change = 5.4, $p = 0.02$, 95% CI: 1.2–6.3) compared to normal (Figure 1). There were no significant differences in expression of COL1 (Fold-change = 8.8, $p = 0.50$, 95% CI: 0.3–10.3) and TGF- β (Fold-change = 1.4, $p = 0.20$, 95% CI: 0.7–1.9) between normal laryngotracheal tissue and scar.

Fibroblasts isolated from normal tracheal mucosa proliferate faster under hypoxic conditions

Demographic and clinical information for the iLTS patients (n=8) who donated fibroblast cell lines are displayed in Table 2. All iLTS cell line donors were adult patients. Cell proliferation of fibroblasts isolated from normal portions of trachea was compared under normoxic and hypoxic conditions (Figure 2). Fibroblasts proliferated significantly faster under hypoxic conditions on all 6 days of incubation ($p < 0.01$ each day).

Fibroblasts isolated from normal trachea show increased expression of IL-6, COL1, α SMA, and MMP13 under hypoxic conditions

Gene expression of fibroblast cell lines isolated from normal trachea was compared under normoxic and hypoxic conditions (Figure 3a). After 12 hours in hypoxia, fibroblasts showed significantly increased expression of IL-6 (Fold-change = 2.6, $p < 0.01$, 95% CI: 1.2–3.2). After 48 hours in hypoxia, fibroblasts showed significantly increased expression of α SMA (Fold-change = 2.0, $p = 0.03$, 95% CI: 1.0–2.7), COL1 (Fold-change = 1.1, $p = 0.03$, 95% CI: 1.0–1.3), and MMP13 (Fold-change = 1.6, $p = 0.01$, 95% CI: 1.1–2.0). TGF- β was significantly decreased after 48 hours under hypoxia (Fold-change = 0.9, $p < 0.01$, 95% CI: 0.8–0.9). At 48 hours under hypoxia, fibroblasts also showed increased protein expression of α SMA (Figure 3b).

Fibroblasts isolated from scarred trachea also proliferate faster under hypoxic conditions, but do not show changes in IL-6, COL1, or α SMA expression

Fibroblasts isolated from laryngotracheal scar (in the same 8 iLTS patients) also displayed significantly faster proliferation under hypoxic conditions compared to normoxic conditions (6 days of incubation, $p < 0.02$ each day) (Figure 4). However, scar fibroblasts did not develop the same expression profile as normal fibroblasts when subjected to hypoxic conditions (Figure 5). TGF- β was significantly decreased after 48 hours under hypoxia (Fold-change = 0.9, $p = 0.01$, 95% CI: 0.8–1.0). No differences in IL-6, MMP13, COL1, α SMA, COL3, or FN1 expression were observed at any of the time points.

DISCUSSION

This is the first study that examines the effect of hypoxia on laryngotracheal fibroblasts and its role in the formation of tracheal scar. Our results lead to several unique perspectives on the pathogenesis of iLTS. While similarities between iLTS and other diseases of fibrosis have always been suspected, this is the first study that demonstrates a myofibroblast phenotype in iLTS scar. Using an *in vitro* model, we show that hypoxia influences both the initiation and maintenance of a fibrotic phenotype, and that these changes may be mediated by inflammatory pathways involving IL-6.

Through our brush biopsies, we demonstrate that the surface cells of laryngotracheal scar display a myofibroblast phenotype. Myofibroblasts can be transdifferentiated from resident fibroblasts and defined by the molecular marker α -smooth muscle actin (α SMA), a protein that gives them twice the contractile force of undifferentiated fibroblasts.²⁵ Under conditions of pathological fibrosis, dysregulated myofibroblasts lay down an abundance of collagens and other ECM proteins.²⁶ Matrix metalloproteinases (MMPs) may also be excessively produced, modifying and maintaining the ECM secreted by myofibroblasts.²⁷ Specifically, upregulation of MMP13 has been shown to play a dysfunctional role in the pathogenesis of IPF.²⁸ With increased expression of α SMA, MMP13, and COL1, our scar brush biopsy results indicate an increased presence of myofibroblasts and a pro-fibrotic phenotype similar to other diseases of fibrosis.

With a better understanding of the differences between healthy mucosa and laryngotracheal scar, we aimed to use an *in vitro* model to explore the role of hypoxia in this phenotypic change. As studies in other organs have used severely hypoxic environments (1% O₂) to induce the transdifferentiation of fibroblasts into myofibroblasts,^{13,14} we expected a similar phenotypic shift in tracheal fibroblasts. Our findings show that when normal tracheal fibroblasts are subjected to hypoxic conditions, they undergo a significant increase in α SMA expression, marking a shift towards a myofibroblast phenotype. We also observe a significant increase in the rate of cell proliferation, and significant upregulation of other pro-fibrotic markers, including MMP13 and COL1. Taken together, these findings support the hypothesis that hypoxia may play a role in the initial phase of iLTS, setting off a cascade of events that includes transdifferentiation of resident tracheal fibroblasts into myofibroblasts and the shift towards a pro-fibrotic phenotype. This is consistent with *in vivo* studies of hypertrophic scars, which show a severely decreased oxygen tension during the proliferative phase of scar formation.²⁰

Our findings also suggest that this phenotypic shift may be related to activation of IL-6, a cytokine involved in both hypoxic and pro-fibrotic pathways. In cardiac myocytes, IL-6 expression can be induced by hypoxic stimulation.²⁹ In keloid fibroblasts, IL-6 expression is significantly higher compared to nonlesional fibroblasts, and inhibition of IL-6 signaling leads to a decrease in ECM production.³⁰ In our brush biopsy experiments, IL-6 expression in iLTS scar is significantly higher compared to healthy tissue. In our *in vitro* experiments, we show that a hypoxic environment leads to a similar upregulation of IL-6 after 12 hours. Interestingly, these inflammatory changes in IL-6 precede the transdifferentiation of fibroblasts into myofibroblasts, which does not begin until 48 hours under hypoxia. While

our results do not prove a causative relationship between IL-6 upregulation and myofibroblast transdifferentiation, we do observe a temporal relationship between the two events. This temporal relationship, taken together with the evidence from other organs, suggests that IL-6 activation may be crucial to the common phenotype observed in fibrotic diseases.

Hypoxia continues to drive increased cell proliferation in tracheal scar, but does not cause the same myofibroblast shift seen in normal fibroblasts. One explanation for this observation is that the fibroblasts isolated from tracheal scar already contain a large population of myofibroblasts. This is evidenced by the significantly increased α SMA expression seen in our scar brush biopsy samples. Thus, hypoxia appears to play a differential role in the formation and maintenance of tracheal scar. This is also consistent with *in vivo* studies of hypertrophic scars, which suggests the low oxygen tension observed in early scar returns to normal in mature scars.²⁰ Once the scar matures, hypoxia no longer appears to drive the inflammatory microenvironment and fibroblast phenotype, but continues to induce the hyperproliferation of fibroblasts. The sustained elevation we observe in cell proliferation under hypoxic conditions may be supported by metabolic adaptations in stressed fibroblasts, which include a lower the rate of oxidative phosphorylation and reduced mitochondrial mass.³¹ This allows fibroblasts to shift its energy production from an oxygen-dependent pathway to an oxygen-independent pathway, creating the Warburg-like effect that we've previously described in our scar fibroblasts.⁹

Our findings open the door to a few therapeutic considerations. Hyperbaric oxygen therapy (HBO) can increase partial oxygen pressure in tissues and address hypoxia, and has been shown to reduce fibrosis after tracheal resection.³² However, more studies are required to better understand HBO's therapeutic effect in the initial phase of tracheal stenosis, when hypoxia appears to have the largest influence on fibroblast transdifferentiation. From an inflammatory standpoint, a humanized antibody to the IL-6 receptor, tocilizumab, has shown early success for the treatment of skin fibrosis.³³ Further animal studies and clinical trials are needed to clarify the therapeutic potential of IL-6 inhibitors in iLTS.

Surprisingly, both normal and scar tracheal fibroblasts showed a significant down-regulation of the pro-fibrotic cytokine TGF- β under hypoxia. Other studies are equivocal; some show that TGF- β stimulation of fibroblasts can lead to transdifferentiation into myofibroblasts,³⁴ while others show that TGF- β production is not increased in fibroblasts under hypoxia.³⁵ In chronically hypoxic wounds, TGF- β signaling pathways may be downregulated,³⁶ suggesting negative feedback mechanisms between hypoxia-induced and TGF- β -induced downstream targets. Future studies may better delineate the interaction between these two signaling pathways.

Although our *in vitro* model is widely used in other organs, it does not fully simulate the inflammatory microenvironment observed *in vivo*. By focusing on fibroblasts, we are able to isolate the effect of hypoxia on these end effector cells in iLTS. However, we cannot comment on how other cytokines and immune cells interact with hypoxia.^{3,5} Evidence from other organs suggests that hypoxia can also lead to increased secretion of myofibroblast

activating cytokines from epithelial cells.³⁷ Contributions from other cell types may be explored in future *in vitro* co-culture experiments.

Finally, we recognize that while our results point to a role for hypoxia in the formation and maintenance of laryngotracheal scar, hypoxia is not likely the sole contributing factor in the pathogenesis of iLTS. As other studies have suggested, additional contributing factors include microbiome shifts in the larynx and trachea³⁸ and the ensuing perichondritis after hypoxic injury caused by intubation.³⁹ Recent discoveries also highlight the potential for a genetic predisposition to laryngotracheal scar formation; one study found an association between a polymorphism in matrix metalloproteinase 1 (MMP1) and the formation of scar after intubation injury.⁴⁰ This discovery complements our finding of elevated MMP activity after hypoxic stimulation of normal laryngotracheal fibroblasts, suggesting that genetic predisposition may enhance the effects of hypoxia on the laryngotracheal ECM. Ultimately, we believe that iLTS is multi-factorial disease arising from a combination of genetic predisposition, hypoxic and mechanical tissue injury, and subsequent consequences on local microbiome and inflammation. Future studies are required to better understand the interaction between these mechanisms and how they all come together to lead to the formation of laryngotracheal scar.

CONCLUSION

Human iLTS scar displays a myofibroblast phenotype, which includes upregulation of α SMA and other pro-fibrotic ECM proteins. Under hypoxic conditions *in vitro*, fibroblasts isolated from normal trachea hyper-proliferate and transdifferentiate into a similar myofibroblast phenotype. This transdifferentiation may be mediated by the fibrosis cytokine IL-6. Fibroblasts from tracheal scar do not display the same transdifferentiation, but do display hyper-proliferation. These results elucidate the differential role of hypoxia in the formation and maintenance of tracheal scar.

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Figure 1. Relative differences in gene expression between brush biopsy samples from normal laryngotracheal tissue and scar (n=9 patients). Expression of IL-6, MMP13, and α SMA are significantly increased in laryngotracheal scar.

Figure 2.
Differences in cell proliferation of *normal* laryngotracheal fibroblasts between hypoxic and normoxic conditions (n=8 patients, p<0.01 for each day)

Figure 3.

Gene a) and protein b) expression of *normal* fibroblasts in normoxia and hypoxia (n= 8). IL-6 is upregulated after 12 hours. COL1, αSMA, and MMP13 are upregulated after 48 hours.

Figure 4. Differences in cell proliferation of fibroblasts from laryngotracheal scar between hypoxic and normoxic conditions (n=8 patients, $p < 0.02$ for each day)

Figure 5.

Comparative gene expression in fibroblasts from laryngotracheal *scar*, under normoxic and hypoxic conditions (n=8). No significant differences are observed in IL-6, COL1, and αSMA expression between the two conditions.

Table 1

Demographic and Clinical History of iLTS Brush Biopsy Donors

Patient	Sex	% Stenosis	Time From Presentation	Cause of Intubation
Patient 1	F	50%	3 years, 10 months	Motor vehicle collision
Patient 2	F	50%	20 years	Laryngeal trauma
Patient 3	F	60%	2 years, 2 months	Diabetic ketoacidosis
Patient 4	F	70%	3 months	Tracheomalacia
Patient 5	F	90%	1 year	Suicide attempt
Patient 6	F	50%	1 year, 7 months	Smoke inhalation
Patient 7	F	75%	4 months	Suicide attempt
Patient 8	M	30%	4 years	Blunt trauma
Patient 9	M	95%	6 years, 11 months	Suicide attempt

Table 2

Demographic and Clinical History of iLTS Cell Line Donors

Patient	Sex	% Stenosis	Time From Presentation	Cause of Intubation
Patient 1	F	50%	2 years, 1 month	Stroke
Patient 2	F	50%	20 years	Motor vehicle collision
Patient 3	F	60%	2 months	Suicide attempt
Patient 4	F	70%	27 years	Neonatal surgery
Patient 5	M	70%	1 year, 9 months	Myocardial infarction
Patient 6	F	50%	1 month	Suicide attempt
Patient 7	F	70%	1 year, 1 month	Respiratory failure
Patient 8	F	80%	5 months	Suicide attempt